

**The Use of Directed Evolution towards Altering the
Substrate Specificity of Acyl-Coenzyme A:
Isopenicillin N Acyl Transferase and Transforming It
from Generalist to Specialist**

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Abstract

Acyl-Coenzyme A: Isopenicillin N Acyl Transferase (AT) is a key enzyme in the biosynthesis of β -lactam antibiotics in penicillin producing organisms such as *P. chrysogenum* and *A. nidulans*. Its natural activity is to exchange the side chain of the low activity antibiotic IPN [18] for the phenylacetyl side chain resulting in the more active antibiotic Penicillin G [5]. The biosynthesis of β -lactams has been exploited towards producing these compounds for therapeutic use. However, increasing bacterial resistance means new analogues in this compound class are constantly sought.

As well as improving current production methods of β -lactam antibiotics, AT's broad substrate specificity means it could potentially play a role in the development and production of alternative β -lactam antibiotics that are able to overcome resistance.

This thesis describes the identification of an AT mutant with improved acylation activity (AAT activity) *via* screening of an AT library using a previously developed screening method. Approaches towards the development of a method for the identification of AT mutants with improved hydrolysis activity were also explored. The main problem to overcome in developing such a screen is the inhibitory effect of 6-APA [1], the product of hydrolysis, on AT's IAT activity. The first approach investigated the potential of increasing the sensitivity of an assay by targeting AT to the periplasm. A second approach using β -lactamases to hydrolyse 6-APA [1] thus freeing up the active site of AT was also investigated.

Preface

Chapter one is a general introduction to the field of β -lactam antibiotics, followed by three results and discussion chapters. Chapter two describes the search for AT mutants with improved AAT activity. Chapters three and four describe two approaches towards the development of screening or selection methods for the identification of AT mutants with improved IAT activity; Chapter three using periplasmic expression of AT and Chapter four using a β -lactamase. Chapters five and six contain information on the experimental methods used in this research. Chapter five details the reagents and equipment used and Chapter six the general techniques employed.

Declaration

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Last but by no means least, my parents without whose support and encouragement this would not have been possible.

Abbreviations

6-APA	6-aminopenicillanic acid
7-ACA	7-aminocephalosporanic acid
7-ADCA	7-aminodeacetoxycephalosporanic acid
α -AAA	L- α -aminoadipic acid
AAT	acyl-coenzyme A:6-aminopenicillanic acid acyltransferase activity
ACS	acetyl-CoA synthetase
ACV	δ -(L- α -aminoadipoyl)-L-cysteinyl-D-valine
ACVS	δ -(L- α -aminoadipoyl)-L-cysteinyl-D-valine synthetase
Adi-NAC	S-adipoyl-N-acetylcysteamine
Amp ^R	Ampicillin resistance gene
<i>A. lactamdurans</i>	<i>Amacolatopsis lactamdurans</i>
<i>A. chrysogenum</i>	<i>Aspergillus chrysogenum</i>
<i>A. nidulans</i>	<i>Aspergillus nidulans</i>
AT	acyl-coenzyme A:isopenicillin N acyltransferase
ATP	adenosine-5'-triphosphate
<i>ato</i>	genes encoding acetyl-CoA:acetate CoA transferase
bp	base pair
BSA	bovine serum albumin
C-	carboxy-
<i>cef</i>	gene involved in cephalosporin biosynthesis
<i>cef D</i>	gene encoding IPN epimerase
<i>cef E</i>	gene encoding DAOCS
<i>cef EF</i>	gene encoding DAOCS/DACS
<i>cef F</i>	gene encoding DACS
<i>cef G</i>	gene encoding DAC acetyltransferase
cam	chloramphenicol
<i>C. acremonium</i>	<i>Cephalosporium acremonium</i>
CFE	cell-free extract
CFU	colony forming unit

<i>cmc</i>	gene involved in cephamycin biosynthesis
CoA	coenzyme A
CoASH	coenzyme A free thiol
Da	Dalton
DAC	deacetylcephalosporin C
DACS	deacetylcephalosporin C synthase
DAOC	deacetoxycephalosporin C
DAOCG	deacetoxycephalosporin G
DAOCS	deacetoxycephalosporin C synthase
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	2'-deoxynucleoside-5'-triphosphate
ds	double-stranded
DTT	dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	ethylenediaminetetra-acetic acid
Hepes	<i>N</i> -hydroxyethylpiperazine- <i>N'</i> -ethanesulfonic acid
¹ H-NMR	proton nuclear magnetic resonance
HPLC	high performance liquid chromatography
hr	hour(s)
IAH	isopenicillin N amidohydrolase activity
IAT	acyl-coenzyme A:isopenicillin N acyltransferase activity
IPN	isopenicillin N
IPNE	isopenicillin N epimerase
IPNS	isopenicillin N synthase
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase
KCLA	potassium clavulanate (clavulanic acid)
kDa	kilo Dalton(s)
<i>N. lactamdurans</i>	<i>Nocardia lactamdurans</i>
MBP	maltose-binding protein
Me	methyl
MIC	minimum inhibitory concentration
min	minutes

<i>M. luteus</i>	<i>Micrococcus luteus</i>
<i>M. lysodeikticus</i>	<i>Micrococcus lysodeikticus</i>
MS	mass spectrometry
N-	amino
<i>N. lactamdurans</i>	<i>Nocardia lactamdurans</i>
NMR	Nuclear magnetic resonance
<i>N. uniformis</i>	<i>Nocardia uniformis</i>
OC-7-ADCA	<i>O</i> -carbamoyl-7-aminodeacetylcephalosporanic acid
OCDAC	<i>O</i> -carbamoyldeacetylcephalosporin C
OCDAG	<i>O</i> -carbamoyldeacetylcephalosporin G
OD ₆₀₀	Optical density at 600 nm
Oligo	Oligonucleotide
PAA	phenylacetic acid
PA-CoA	phenylacetyl-coenzyme A
PA-NAC	<i>S</i> -phenylacetyl- <i>N</i> -acetylcysteamine
PAGE	polyacrylamide gel electrophoresis
PBP	penicillin binding protein
<i>P. chrysogenum</i>	<i>Penicillium chrysogenum</i>
<i>P. notatum</i>	<i>Penicillium notatum</i>
PCR	polymerase chain reaction
<i>penDE</i>	gene encoding AT
pen G	penicillin G
pen N	penicillin N
pen V	penicillin V
<i>P. aeruginosa</i>	<i>Pseudomonas aeruginosa</i>
<i>P. fluorescens</i>	<i>Pseudomonas fluorescens</i>
recAT	recombinant AT
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
<i>S. cattleya</i>	<i>Streptomyces cattleya</i>
<i>S. clavuligerus</i>	<i>Streptomyces clavuligerus</i>
<i>S. pyogene</i>	<i>Streptomyces pyogene</i>
SDS	sodium dodecyl sulphate
spp.	species
ss	single stranded

TEMED	<i>N,N,N',N'</i> -tetramethylethylenediamine
TLC	thin layer chromatography
Tris-HCl	tris(hydroxymethyl)aminomethane hydrochloride
T _m	melting temperature
T _s	transition
T _v	transversion
UV	ultraviolet
V	volts
wt	wild type

Symbols for Nucleic and Amino Acids

Nucleic acids

A	adenine
C	cytosine
G	guanine
T	thymine
N	A, C, G or T.

Amino acids

Amino Acid	Three Letter Code	One Letter Code
Alanine	Ala	A
Cysteine	Cys	C
Aspartic acid	Asp	D
Glutamic acid	Glu	E
Phenylalanine	Phe	F
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Lysine	Lys	K
Leucine	Leu	L
Methionine	Met	M
Asparagine	Asn	N
Proline	Pro	P
Glutamine	Gln	Q
Arginine	Arg	R
Serine	Ser	S
Threonine	Thr	T
Valine	Val	V
Tryptophan	Trp	W
Tyrosine	Tyr	Y

The reasonable man adapts himself to the world; the unreasonable one persists in trying to adapt the world to himself. Therefore all progress depends on the unreasonable man.

G.B. Shaw

CHAPTER ONE

Introduction

1.1 The Discovery and Development of β -Lactam Antibiotics

β -lactam antibiotics are the most widely used chemotherapeutic compounds in use today and their discovery and development is one of the major advances of modern times. Since their discovery mortality rates due to previously fatal bacterial infections have fallen, and without them the modern surgery that we now take for granted would not be possible.

Penicillin was initially discovered in Oxford by Alexander Fleming in 1929 (Fleming 1929) and he recognized its potential in the use of surgical dressings. However, it was not until the early 1940's that its *in vivo* antibacterial activity was demonstrated (Chain 1940; Abraham 1941). Subsequent development and mass production of Penicillin was achieved by collaborations between British and American research institutions and industry, largely driven by the need created by World War II (Abraham 1990). These efforts were rewarded in 1945 with the Nobel Prize in Physiology or Medicine being awarded jointly to Sir Alexander Fleming, Ernst Boris Chain and Sir Howard Walter Florey "*for the discovery of penicillin and its curative effect in various infectious diseases*".

In 1945 the structure of Penicillin was elucidated (Crowfoot 1948) and this information enabled the total synthesis of Penicillin which was achieved by 1950 (Sheehan and Henerylogan 1962). However, this method was not suitable for the large-scale production of β -lactams.

In 1957 the penicillin nucleus, 6-aminopenicillanic acid (6-APA) [1] was isolated from fermentation broths (Batchelor, Doyle et al. 1959) which allowed the production of semi-synthetic β -lactams and the first chemotherapeutic semi-synthetic penicillin, Methicillin [2] was introduced in 1960 (Rolinson and Geddes 2007). The widespread use and misuse of penicillins lead to the emergence of bacterial resistance and other semi-synthetic analogs were designed and developed in order to overcome this concern; this race against bacterial resistance still continues.

Work towards the cephalosporin β -lactam antibiotics followed a similar path. A cephalosporin producing strain was first discovered by Guiseppe Brotzu in 1945. Subsequent identification of Cephalosporin C [3] (Newton and Abraham 1954) followed. However, the corresponding cephalosporin nucleus, 7-aminocephalosporanic

acid (7-ACA) [4] was not so easy to obtain by fermentation methods therefore semi-synthetic analogues were not so forthcoming. In 1961 Lilly Research labs developed a method of chemically expanding the five-membered ring in Penicillin G [5], the deacylation of which, lead to the cephalosporin nucleus (Morin, Roeske et al. 1962). The structure of Cephalosporin C [3] was elucidated and in 1964 the Nobel Prize in Chemistry was awarded to Dorothy Crowfoot Hodgkin *"for her determinations by X-ray techniques of the structures of important biochemical substances"*.

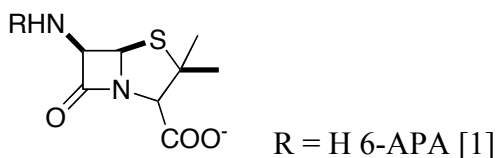
The bulk production of β -lactam antibiotics is a marvelous example of biotechnology and as a result they are commodity chemicals unlike other pharmaceuticals. However, increasing bacterial resistance means the continual development of these clinically vital compounds is essential if we are to enjoy our current standards of healthcare.

1.2 β -Lactam Antibiotic Classes

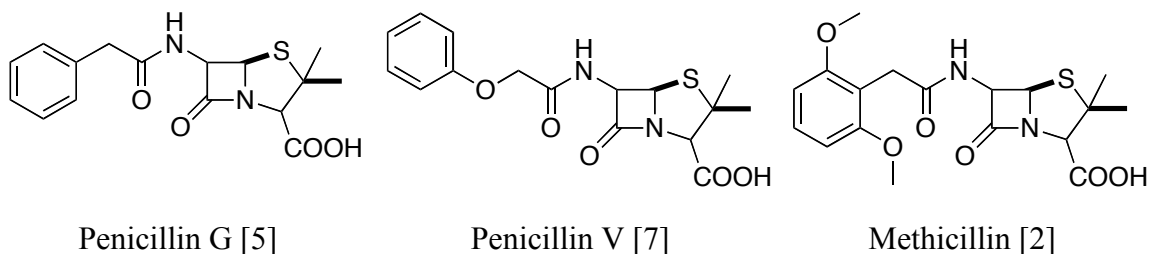
There are many β -lactam antibiotics in use today. Their common structural feature is the highly reactive four-membered β -lactam ring. An ever-increasing class of compounds, classification is primarily based on their structures with further subdivision based on their bacterial range of action. They can be described in terms of a β -lactam nucleus with a side chain. Different combinations result in different properties for example amoxicillin [6] is orally stable whereas Penicillin G [5] is not.

1.2.1 Penicillins

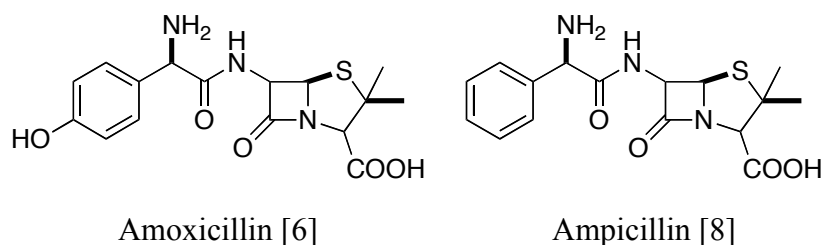
Penicillins are β -lactams that contain 6-APA [1] as the β -lactam nucleus. This consists of a five-membered thiazolidine ring fused to the four-membered β -lactam ring. They are generally classified according to their range of action narrow- medium- broad or extended-spectrum. Some examples are shown in Figure 1.1.



Narrow spectrum



Moderate spectrum



Extended spectrum

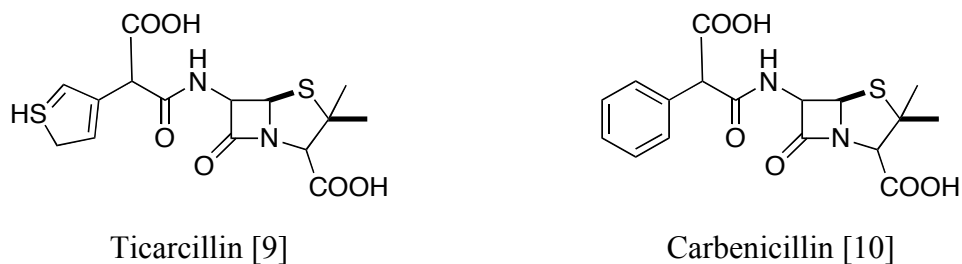


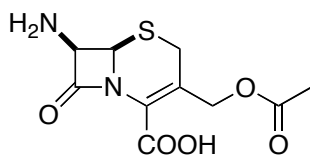
Figure 1.1 Examples of penicillins

1.2.2 Cephalosporins

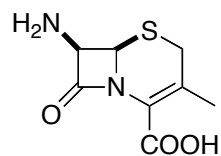
Cephalosporins are β -lactams that contain either 7- aminocephalosporanic acid, 7-ACA [4] or 7-aminodeacetoxycephalosporanic acid, 7-ADCA [11] as the β -lactam nucleus. They differ to penicillins in that a six-membered dihydrothiazine ring is fused to the four-membered β -lactam ring. They are generally classified by generations, which like the penicillins are based on their range of action and also their time of development. Some clinically useful examples are shown in Figure 1.2.

Cephalosporin nuclei

7-ACA [4]

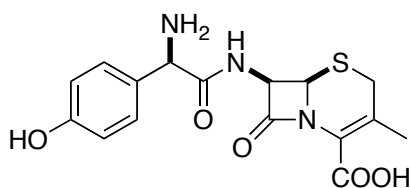


7-ADCA [11]

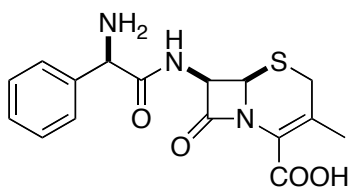


Cephalosporins

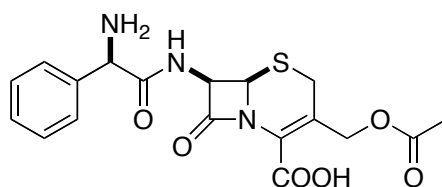
Cefadroxil [12]



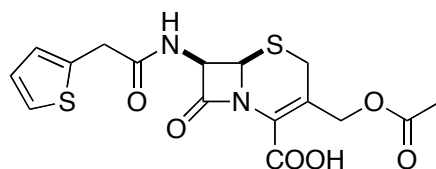
Cephalexin [13]



Cephaglycine [14]



Cephalothin [15]



Cefamandole [16]

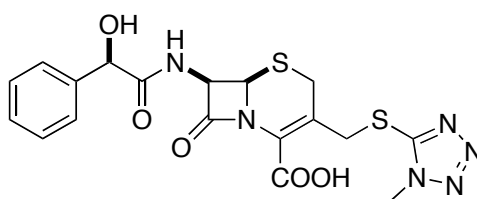


Figure 1.2 Examples of cephalosporins

1.2.3 Other Classes of β -Lactams

Other classes of β -lactams contain a similar fused five-membered ring like penicillins, however, in place of the sulfur there is a carbon (*e.g.* thienamycin [16]), an oxygen (*e.g.* clavulanate [17]) or no second ring at all *i.e.* monobactams. (Shown in Figure 1.3).

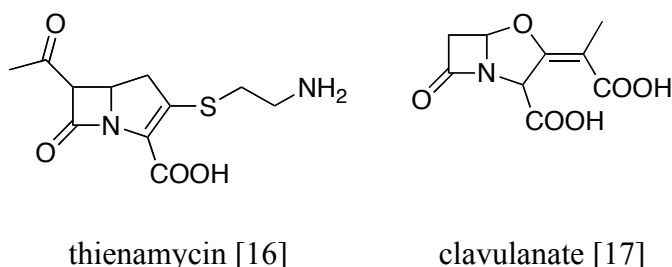


Figure 1.3 Other classes of β -lactam antibiotics

1.3 Mode of action

How β -lactam antibiotics actually work in treating bacterial infections has been studied since the discovery of penicillin. An understanding of their mechanism allows the design of more efficient, broad range antibiotics, the need for which is ever increasing as bacterial resistance continues apace.

Initial clues as to how they might work came from the observation of various effects of Penicillin G on bacteria. *E. coli* could be grown in the presence of Penicillin G [5] in hypertonic media. The resulting protoplasts did not lyse in the medium but did lyse when transferred to water. It was also observed that these protoplasts did not form when penicillin was present but the requirements for growth were not (Lederberg 1956). Penicillin was also found to only kill growing cells (Hurwitz, Reiner et al. 1958) and finally, Gram-negative bacteria are less susceptible to penicillin than Gram-positive bacteria.

It was later shown that penicillin interferes with a cross linking transpeptidation reaction during the last stage of bacterial cell wall formation and it was proposed that penicillin is an analogous substrate for the transpeptidase enzyme responsible for this reaction (Tipper and Stroming, 1965; Wise and Park 1965).

The cell wall is made up of peptidoglycan, which consists of linear polysaccharide chains cross-linked by pentapeptides. The polysaccharide chains are made up of

alternating amino sugar units *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) linked *via* β -1, 4 linkages. The pentapeptides are attached to the MurNAc sugar units and their amino acid sequence is usually, though not always, L-Ala- γ -D-Glu-X-D-Ala-D-Ala where X is generally meso-diaminopimelate (*m*-DAP) in Gram-negative bacteria and L-Lys in Gram-positive bacteria (Bugg and Walsh 1992) (Figure 1.4).

Cross-links between the polysaccharide chains are formed by transpeptidation reactions between the polypeptides attached to MurNAc. At the terminus of these polypeptide chains are D-amino acids and it is these that penicillins are structurally analogous to.

Penicillins inhibit transpeptidation by binding irreversibly to transpeptidases *via* a covalent bond formed between a serine residue on the enzyme and the carbonyl carbon of the β -lactam ring. For this reason, these enzymes are also known as penicillin binding proteins (PBPs).

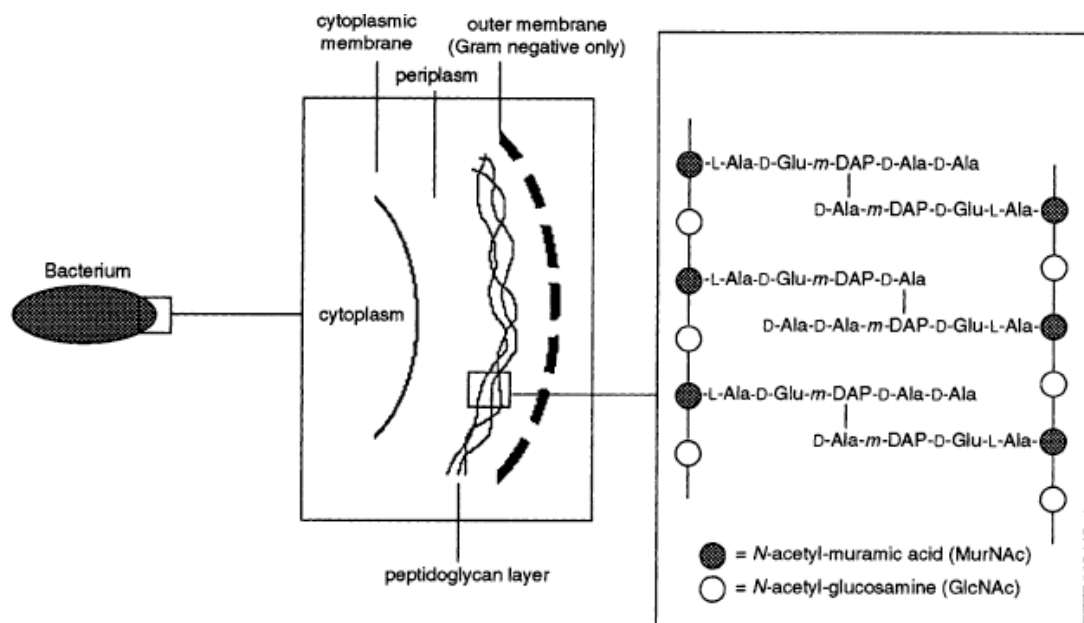


Figure 1.4 Simplified structure of peptidoglycan (Bugg and Walsh 1992).

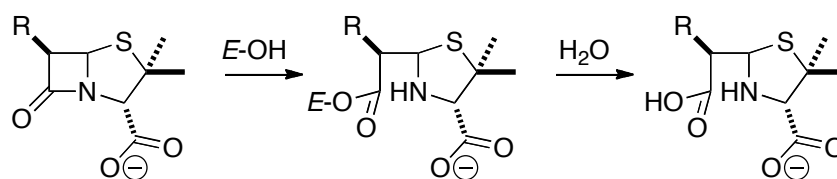
The reduction in cross-linkages means that the cell wall is not as rigid and the cell succumbs to osmotic pressure and dies. Fortunately there are no equivalent enzymes in human biosynthesis therefore penicillins specifically target bacteria, which is why they are so clinically useful.

1.4 Bacterial Resistance

Although effective at inhibiting peptidoglycan biosynthesis in bacteria the widespread use and misuse of β -lactam antibiotics has allowed bacteria to evolve in order to overcome their effects. There are four primary mechanisms by which bacteria can resist β -lactam antibiotics (Babic, Hujer et al. 2006; Drawz and Bonomo 2010) and these are detailed briefly below.

1.4.1 Production of β -Lactamases

This is the most common and important mechanism of resistance in Gram-negative bacteria. β -lactamases, as their name suggests, inactivate β -lactam antibiotics by hydrolyzing the amide bond of the β -lactam ring (§ Scheme 1.1). The first β -lactamase was observed around the same time as β -lactam antibiotics were themselves undergoing initial discovery and development (Abraham 1940).



Scheme 1.1 To show the hydrolysis of a β -lactam by a β -lactamase

There are currently two approaches towards overcoming the resistance inferred to bacteria by constantly evolving β -lactamases. One is the discovery and design of different β -lactam antibiotics that are not so readily hydrolysed by β -lactamases. The other is the development of suitable β -lactamase inhibitors. These compounds are also β -lactams and they inhibit by binding irreversibly to β -lactamases thus preventing them from hydrolyzing the β -lactam antibiotics they are used in conjunction with. However, due to the rapid rate of β -lactamase evolution this is a constant challenge, made all the more difficult by their necessary exposure to a wide variety of chemical agents.

The potential use of β -lactamases was investigated in this project and further discussion can be found in Chapter 4.

1.4.2 Changes in the Active Site of PBPs

As discussed above (§ 1.3) PBPs (or transpeptidases) play a role in cell wall formation and are inhibited by irreversible binding of β -lactam antibiotics. Changes in the active site lower the affinity for β -lactam antibiotics thus increasing resistance to these agents. Examples in pneumococci have been recorded (Laible, Spratt et al. 1991).

1.4.3 Decreased Expression of Outer Membrane Proteins

Outer membrane proteins in bacterial cell walls allow substances *e.g.* β -lactam antibiotics to permeate the cell therefore any changes in their expression or structure can lower their permeability. However, this is usually not enough on its own to affect resistance and these mutants are often found in combination with β -lactamases (Douthett, Ellington et al. 2009).

1.4.4 The Use of Efflux Pumps

Efflux pumps are also proteins in the cell membrane and are capable of exporting substances from the cell. In addition to altered cell permeability and β -lactamases they are another mechanism bacteria can evolve to improve their ability to resist β -lactam antibiotics.

Despite the growing problem of resistance to β -lactam antibiotics they are still the drug of choice due to their low cost and low toxicity and their development continues. Current efforts focus on improving production methods and exploring the potential of alternative β -lactam antibiotics that can be created from various combinations of nucleus and side chains. In order to develop alternative β -lactam antibiotics an understanding of how they are produced in organisms has been the subject of much study. This information has made possible the manipulation of these organisms towards producing analogous compounds and optimizing production methods.

1.5 The Biosynthesis of β -Lactam Antibiotics

1.5.1 The Biosynthetic Pathway

The sulfur containing penicillins and cephalosporins are the focus of this work and their biosynthesis will be discussed. Biosynthesis starts from the same amino acid precursors for both penicillins and cephalosporins. The initial steps in both routes are towards the synthesis of Isopenicillin N (IPN) [18] after which the routes for penicillins and cephalosporins then necessarily differ. The details are discussed below (Martín 2010).

The formation of IPN [18] is carried out in two steps starting from the three amino acids L- α -aminoadipic acid [19], L-cysteine [20] and L-valine [21] using 2 enzymes, ACV synthetase (ACVS) and IPN synthase (IPNS). L- α -aminoadipic acid [19] is a non-proteinogenic amino acid formed from lysine.

ACVS takes the three amino acids, L- α -aminoadipic acid [19], L-cysteine [20] and L-valine [21] and forms the tripeptide, L- δ -(α -aminoadipyl)-L-cysteinyl-D-valine (ACV) [22]. After initial activation of the amino acids with ATP, the resulting aminoacyl-adenylates are bound to the enzyme as thioesters. Valine is then epimerized from the L- to the D-isomer by ACVS. The enzyme then links all three aminoacyl-adenylates to form ACV [22] which is then released *via* internal thioesterase activity (Martin 2000). ACV synthetases are multifunctional proteins as demonstrated by this sequence of events. They are encoded by the *pcbAB* gene, which occurs in fungal and bacterial penicillin and cephalosporin gene clusters.

The next step in the pathway is carried out by IPNS. IPNS takes the tripeptide ACV [22] and removes four protons, subsequent cyclisation to form the penam nucleus results in the formation of IPN [18], itself weakly antibiotic. IPNS is encoded by the *pcbC* gene.

Once IPN [18] is formed it is here that the pathways for penicillins and cephalosporins diverge.

For the biosynthesis of penicillins, the next and final step after IPN [18] formation is carried out by an acyltransferase (AT) encoded by the *penDE* gene. Hydrolysis of the α -aminoadipoyl side chain of IPN [18] by the acyltransferase results in the formation of the penicillin nucleus, 6-APA [1], which is then re-acylated with an acyl-Co-A

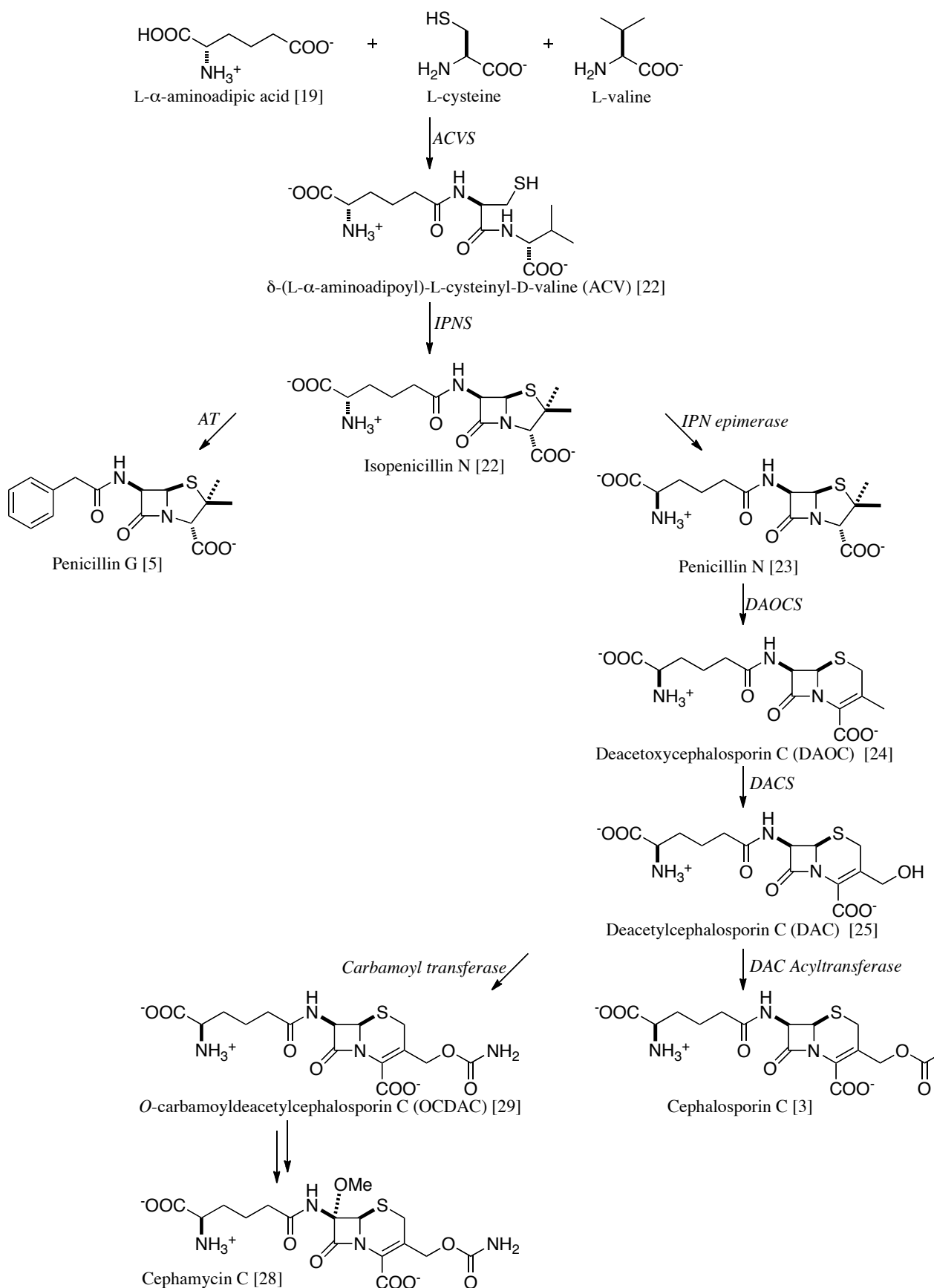
derivative as acyl donor to give the corresponding penicillin. For example acylation with phenylacetic acid gives Penicillin G [5]. The *penDE* gene is not present in cephalosporin producing organisms. Acyltransferase (AT) is the subject of this study.

Cephalosporin biosynthesis continues from IPN [18] as follows. Epimerization of IPN [18] (L-isomer) to Penicillin N [23] (D-isomer) is carried out by an IPN epimerase encoded by two linked genes, *cefD1* and *cefD2*. The five-membered thiazolidine ring is then enzymatically expanded to the six-membered dihydrothiazine ring by deacetoxycephalosporin C (DAOC) synthase. IPN [18] is a poor substrate for this enzyme, as are Penicillin G [5] and 6-APA [1]. However, adipyl- (Adi-Pen [26]) and glutaryl-6-APA [27] derivatives are accepted (Wu, Fan et al. 2005).

The next step in the biosynthesis is hydroxylation at C-3 to form deacetylcephalosporin C (DAC) [25]. This can be done in two ways depending on the organism. In *A. chrysogenum* DAOC synthase hydroxylates after chemical expansion of the ring. However, in bacteria there is a separate hydroxylase enzyme present encoded by the *cefF* gene.

In *C. acremonium* the final step in the biosynthesis is carried out by DAC acyltransferase, encoded for by the *cefG* gene, which uses acetyl Co-A as an acetyl group donor; this results in the formation of Cephalosporin C [3].

In *S. clavuligerus* and *N. lactamdurans*, Cephalosporin C is not produced, as there is no DAC acyltransferase. Instead, carbamoylation at C-3 and hydroxylation and methylation at C-7 results in Cephameycin C [28]. These reactions are carried out by a DAC-carbamoyl transferase, 7'- α -hydroxylase and methyltransferase enzymes encoded by *cmcH*, *cmcI* and *cmcJ* genes respectively (Martin, Gutierrez et al. 1994). The overall route towards these compounds is shown in Scheme 1.2.



Scheme 1.2 To show the biosynthetic pathway of β -lactams

1.5.2 β -Lactam Biosynthetic Genes

The genes involved in the biosynthesis of β -lactam antibiotics in various organisms have been extensively studied and reviewed. (Ingolia and Queener 1989; Martin, Gutierrez et al. 1994). The nomenclature used is as follows; *pcb* corresponds to genes involved in the synthesis of penicillins and cephalosporins, *pen* indicates genes for penicillins only, *cef* for cephalosporins and *cmc* for cephamycins. Many of the genes encoding enzymes involved in the biosynthesis of β -lactams have been cloned and sequenced towards the goal of further understanding the production of these compounds. Importantly for this study, the *penDE* gene from both *P. chrysogenum* (Barredo, Vansolingen et al. 1989; Tobin, Fleming et al. 1990) and *A. nidulans* (Tobin, Fleming et al. 1990) have been determined. A comparison of the corresponding amino acid sequences can be found in Appendix E.

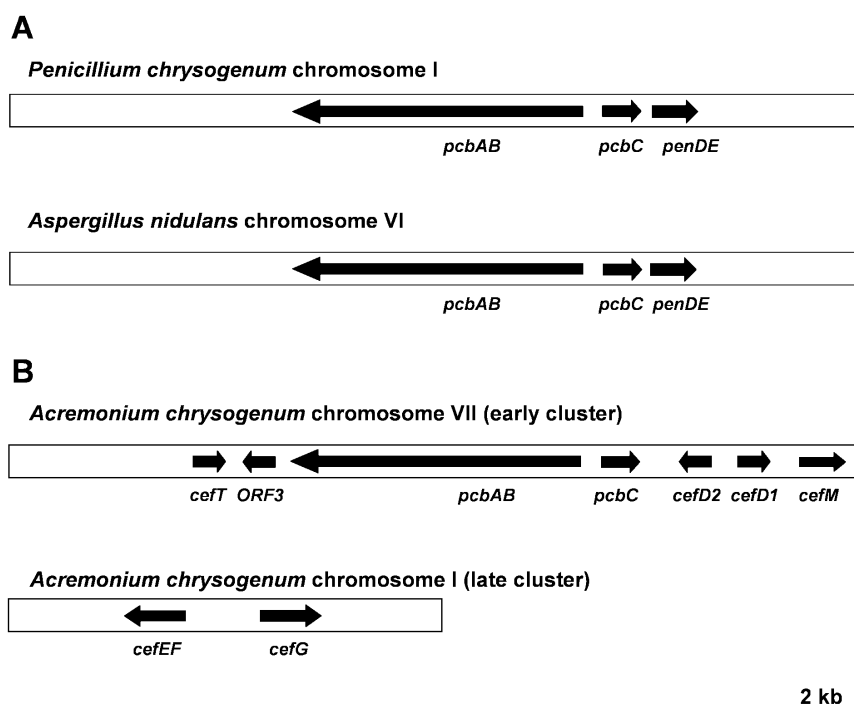


Figure 1.5 A) Penicillin gene clusters in *P. chrysogenum* and *A. nidulans*
B) Cephalosporin gene clusters in *A. chrysogenum*

1.5.3 β -Lactam Producing Organisms

A variety of organisms produce β -lactam antibiotics. Filamentous fungi are able to produce penicillins and cephalosporins but not clavams, carbapenams or monobactams. Bacteria make cephalosporins, cephamycins, clavams, carbapenams and monobactams but not penicillins. This is summarized in Table 1.1 (Martin and Gutierrez 1995).

Class of β -lactam	Fungi	Bacteria	
		Gram-negative	Gram-Positive
Penicillins	<i>Aspergillus</i>		
	<i>Penicillium</i>		
	<i>Epidermophyton</i>		
	<i>Trichophyton</i>		
	<i>Polypaecilum</i>		
	<i>Malbranchea</i>		
	<i>Sartorya</i>		
	<i>Pleurophomopsis</i>		
Cephalosporins and	<i>Cephalosporium</i>	<i>Streptomyces</i>	<i>Flavobacterium</i>
Cephamycins	<i>Anixiopsis</i>	<i>Nocardia</i>	<i>Xanthomonas</i>
	<i>Arachnomycetes</i>		<i>Lysobacter</i>
	<i>Spiroidium</i>		
	<i>Scopulariopsis</i>		
	<i>Diheterospora</i>		
	<i>Paecilomyces</i>		
Clavams		<i>Streptomyces</i>	
Carbapenams		<i>Streptomyces</i>	<i>Serratia</i>
			<i>Erwinia</i>
Monobactams		<i>Nocardia</i>	<i>Pseudomonas</i>
			<i>Gluconobacter</i>
			<i>Chromobacter</i>
			<i>Agrobacter</i>
			<i>Acetobacter</i>

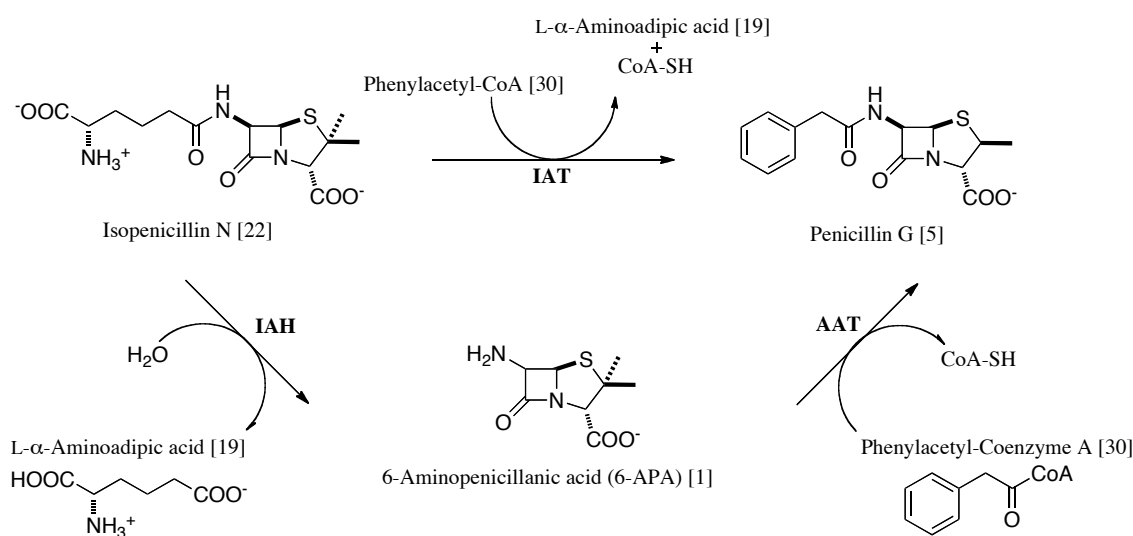
Table 1.1 To show the β -lactams produced by various microorganisms

1.6 Acyl Coenzyme A: Isopenicillin N Acyltransferase (AT)

1.6.1 Introduction

As acyl coenzyme A: Isopenicillin N acyltransferase (AT) is the focus of this project, more detail on this enzyme is now provided.

As has been discussed, AT catalyses the final step in the biosynthesis of Penicillin G [5] in *P. chrysogenum* and *A. nidulans*. Penicillin G [5] may be formed by direct transacylation (IAT) of IPN [18] or by a two-step reaction involving amidohydrolase (IAH) and acyltransferase (AAT) activities with 6-APA [1] as the intermediate. The α -aminoadipic acid side chain of IPN [18] is exchanged for coenzyme A activated phenylacetic acid as shown in Scheme 1.3 (Alvarez, Cantoral et al. 1987; Whiteman, Abraham et al. 1990). It can accept a wide variety of side chains, hydrophobic and hydrophilic acyl-CoA derivatives and some non CoA thioesters as acyl donors (Alonso, Bermejo et al. 1988; Ferrero, Reglero et al. 1991).



Scheme 1.3 To show the reactions catalysed by AT

Mature AT is an α , β -heterodimeric protein containing 357 amino acids with 11kDa and 29kDa subunits. It is produced as an inactive 40kDa proenzyme precursor which is

autocatalytically activated by post translational cleavage of the peptide bond between Gly102 and Cys 103.

1.6.2 Initial Identification and Characterisation

Initial reports on the production of AT believed it to be a monomeric protein (Alvarez, Cantoral et al. 1987). However further characterization studies showed it to be a 40 kDa α,β -heterodimer, which undergoes post-translational cleavage of the Gly102-Cys103 peptide bond resulting in α and β units of 11 and 29 kDa respectively. (Tobin, Fleming et al. 1990; Aplin, Baldwin et al. 1993; Aplin, Baldwin et al. 1993). Further investigations into the requirements for proenzyme cleavage into mature AT were done by introducing amino acid substitutions into the cleavage site, confirming the importance of Cys103 at the *N*-terminus of the 29kDa β -subunit for cleavage and activity (Tobin, Cole et al. 1995).

It was found that both subunits are required for the production of active AT. Co-expression of plasmids encoding the α and β units in recombinant *E. coli* results in correctly folded active enzyme. Expression of the units separately and subsequent mixing does not result in active AT; active AT is only achieved when the subunits are refolded in the presence of urea (Tobin, Baldwin et al. 1993).

The development of a high level *E. coli* expression system for the *penDE* gene of *P. chrysogenum* (Aplin, Baldwin et al. 1993) and the resulting availability of recombinant AT has allowed its purification for mechanistic and structural studies.

1.6.3 *penDE* gene

The *penDE* gene from both *P. chrysogenum* (Barredo, Vansolingen et al. 1989; Tobin, Fleming et al. 1990) and *A. nidulans* (Tobin, Fleming et al. 1990) have been determined. A comparison of the corresponding amino acid sequences can be found in Appendix E. Entire genome sequencing of *P. chrysogenum* has also been carried out and a paralog of *penDE* was found. However under the conditions employed for the study, the gene did not transcribe therefore its actual function was not revealed (van den Berg, Albang et al. 2008). Further studies revealed this paralog Pc13g09140 to express a protein highly similar to AT. However, no penicillin biosynthesis activity was detected even when targeted to the microbodies (Garcia-Estrada, Vaca et al. 2009).

1.6.4 Localization of AT to Microbodies

The various steps in the biosynthesis of penicillins occur in different parts of the cell allowing each enzyme involved in the process to operate under its optimal conditions. It has been shown that AT is localized to the microbodies of *P. chrysogenum* and its location there is important for penicillin production. Microbodies are small organelles (200-800 nm) surrounded by a single membrane. Using site directed mutagenesis to remove the signal sequence (AlaArgLeu) required for targeting the protein to the microbodies, it was shown that although active AT was produced only IPN [18] was synthesized and not Penicillin G [5] (Muller, Bovenberg et al. 1992). It has also been observed that high penicillin producing strains have a high volume of microbodies present (van den Berg, Albang et al. 2008). However, in *A. nidulans* it has been shown that localization of AT in the peroxisomes is not essential for penicillin production (Sprote, Brakhage et al. 2009). These differences between *P. chrysogenum* and *A. nidulans* are important to be aware of as *A. nidulans* is generally used as a model organism and *P. chrysogenum* for production. It has been pointed out that β oxidation take place in the microbodies, the products of which are aliphatic CoA adducts, substrates for AT (Bokhove, Yoshida et al. 2010).

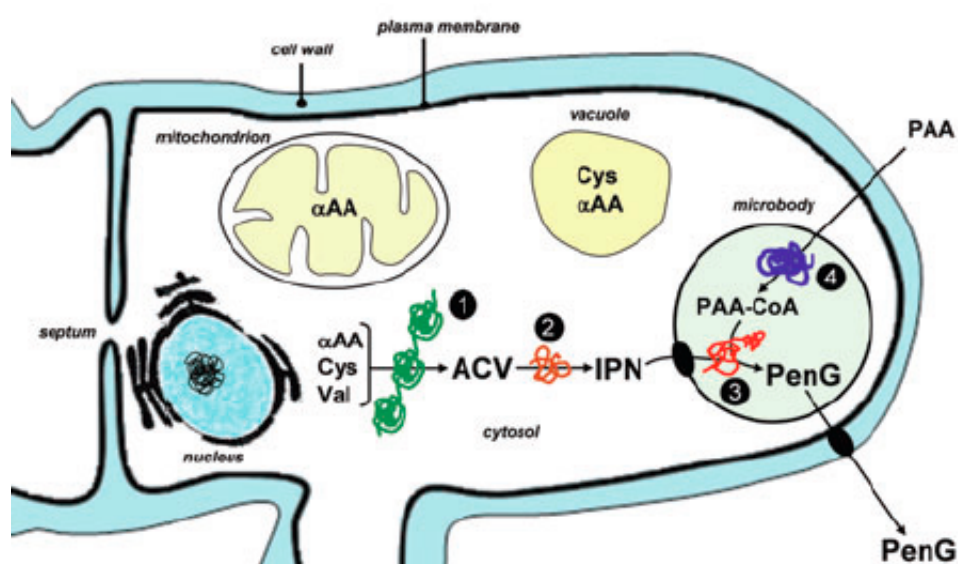


Figure 1.6 Compartmentalization of the penicillin biosynthetic pathway. 1) ACV synthetase; 2) IPN synthase; 3) AT; 4) Phenylacetyl-CoA ligase (Martín 2010).

1.6.5 Structural Determination of AT

To understand how AT catalyzes the acyltransferase reaction and recognizes various substrates its crystal structure is highly desirable. Although recombinant technology has allowed the production of pure AT, obtaining pure homogenous preparations is difficult due to the propensity of the mature form to aggregate. This in turn makes the preparation of crystals for study by x-ray difficult.

However, this problem was circumvented with the use of the mutant Cys103Ala, which can be purified as a monodisperse protein in large yields. This corresponds to the proenzyme form of the enzyme as autocatalytic cleavage between Gly102 and Cys103 does not take place. This proenzyme form of AT was purified and crystallized and was the first x-ray study on AT (Hensgens, Kroezinga et al. 2002).

In another approach, the use of additives to prevent aggregation was investigated using dynamic light scattering (DLS). It was found that incubation of purified AT with 5 mM DTT + 250 mM NaCl + 5 mM EDTA was effective in preventing aggregation. AT was successfully crystallized and crystals diffracting up to 1.64Å resolution were obtained (Yoshida, Hensgens et al. 2005).

Although the crystal structures of both the proenzyme (Cys103Ala mutant) and mature enzyme have been achieved, precise modelling of the substrate in the active site of AT has not been possible due to the difficulty in obtaining a substrate-AT complex for crystallographic studies. However, a suggested substrate binding model based on the proenzyme Cys103Ala crystal structure has been proposed by this group and is discussed in Chapter 2 (§ 2.2).

More recent modeling to further investigate the structure and functioning of AT by comparing the proenzyme (Cys103Ala mutant) and the mature enzyme has been carried out and is now discussed (Bokhove, Yoshida et al. 2010).

AT belongs to the Ntn-hydrolase (*N*-terminal nucleophile aminohydrolases) superfamily. Structures of this class have either serine, threonine or cysteine as the *N*-terminal nucleophile and share similar folding patterns. They consist of four layers of α -helices and β -sheets ($\alpha\beta\beta\alpha$ motif) and have equivalent stereochemistry at the active site. In the case of AT cysteine is the *N*-terminal nucleophile and although it does not

show obvious sequence homology with other enzymes of this class, it does share the structural $\alpha\beta\alpha$ motif.

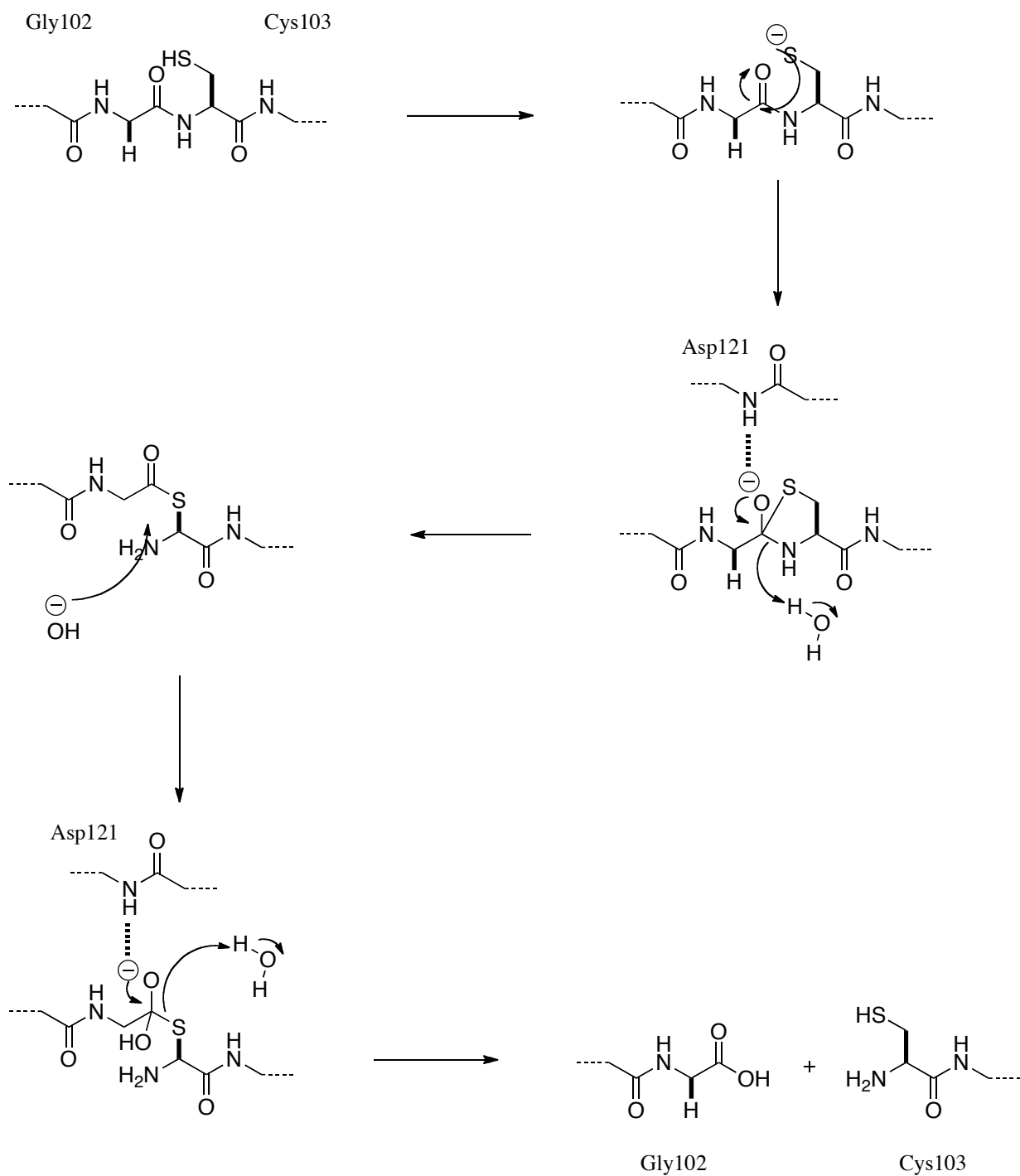
The residues required for autoproteolysis and catalysis were identified by comparing the precursor structure with a tetrahedral reaction intermediate model. Interestingly the same residues are involved for both processes but they perform different roles.

1.6.5.1 The Autoproteolysis Mechanism

In order to obtain further insight into the mechanism of the autocatalytic cleavage of AT the residues around the cleavage site were investigated. The structure of the proenzyme Cys103Ala mutant showed that the peptide bond between Gly102 and Cys103 was inaccessible to a protease or solvent indicating that activation is *via* an intramolecular catalytic event.

Possible candidates to act as the nucleophile for such an event were considered. A water molecule is present in the vicinity however the lack of a catalytic base to activate it eliminated this as a possibility. Intramolecular attack by Cys103 was deemed more likely. Cys103 is believed to be in the correct conformation due to the tight packing of the surrounding residues, allowing it to attack the scissile carbonyl bond. Although in this case there is also no suitable base to activate the nucleophile, when the nucleophilicity of cysteine compared to serine or threonine was considered it was suggested that solvent would be sufficient to deprotonate Cys103 and indeed a suitable water channel was found to exist. The resulting thiolate ion may then attack the carbonyl of Gly102.

Collapse of the tetrahedral intermediate by protonation of the leaving group amine of Cys103 by a water molecule, results in a thioester intermediate, which is then attacked by the hydroxide ion to form a second tetrahedral intermediate. Finally, this second tetrahedral intermediate collapses on protonation of the sulfur atom of Cys103 by another water molecule, which results in free C- and N- termini and overall cleavage of the Gly102-Cys103 peptide bond. The backbone amide bond of Asp121 is in the correct position to stabilize the tetrahedral intermediates in both cases. This sequence of events is shown in Scheme 1.4.



Scheme 1.4 To show the mechanism of autocatalytic cleavage of Gly102-Cys103

1.6.5.2 Substrate Binding in AT and Mechanistic Implications

In terms of identifying the active site and key residues for binding of substrates, AT was compared to enzymes that carry out similar reactions with similar substrates, namely cephalosporinase (CA) and Penicillin G acylase (PGA). CA and PGA also belong to

the Ntn-hydrolase superfamily and like AT can cleave the side chains from β -lactams, 7-ACA [4] in the case of CA and 6-APA [1] in the case of PGA.

In the precursor mimic Cys103Ala, the active site is blocked by residues 95 through to 102. However, in the mature enzyme these residues were found to have moved, revealing a large binding site, much larger than that of CA or PGA. Co-crystallisation of the mature enzyme with 6-APA [1] showed it to bind near Cys103. Van der waals interaction of the thiazole ring with Phe122 and Phe123 was seen which correlated with the postulated binding proposed by this group discussed in Chapter 2 (§ 2.2). A salt bridge between the carboxylate of 6-APA [1] and Arg310 was revealed.

Co-crystallisation of IPN [18] was not possible. However modeling based on the binding and coordination of Penicillin G in PGA led to a transition model resulting from nucleophilic attack of the sulfur atom of Cys103. The key residues proposed are summarized in Table 1.2 below.

Residues	Comments
Trp120	Undergoes conformational change in order to allow the α -aminoadipic acid side chain to bind.
Lys154	H-bonding with carboxylate of α -aminoadipic acid.
Gln118	H-bonding with amino of α -aminoadipic acid.
Ala168	Forms part of oxyanion hole for carbonyl oxygen of scissile bond.
Asn246	Forms part of oxyanion hole for carbonyl oxygen of scissile bond.
Asp121	Transition state stabilization.
Lys154, Tyr166, His182	Highly flexible residues which line the substrate binding site.
Leu130, Ile146, Leu314, Phe315	Hydrophobic side chains in the active site exposed by rotation of Trp120.
Thr126, Glu148	Hydrophilic side chains in the active site exposed by rotation of Trp120.

Table 1.2 Summary of key residues identified by AT modeling

Notable, is the presence of hydrophobic and hydrophilic residues in the active site which goes towards explaining how AT has such broad substrate specificity and can

accept such chemically diverse side chains such as the polar α -aminoadipic acid and the apolar phenylacetyl.

It was also proposed that AT has a storage pocket for 6-APA [1]; 6-APA [1] relocates here to allow the α -aminoadipic acid side chain to leave the active site as shown in Figure 1.7. This proposal is a further extension of the simple mechanism put forward by previous group member Darren Hart which proposed the idea of two binding pockets, one for the penicillin nucleus and one for the side chain (Hart 1996).

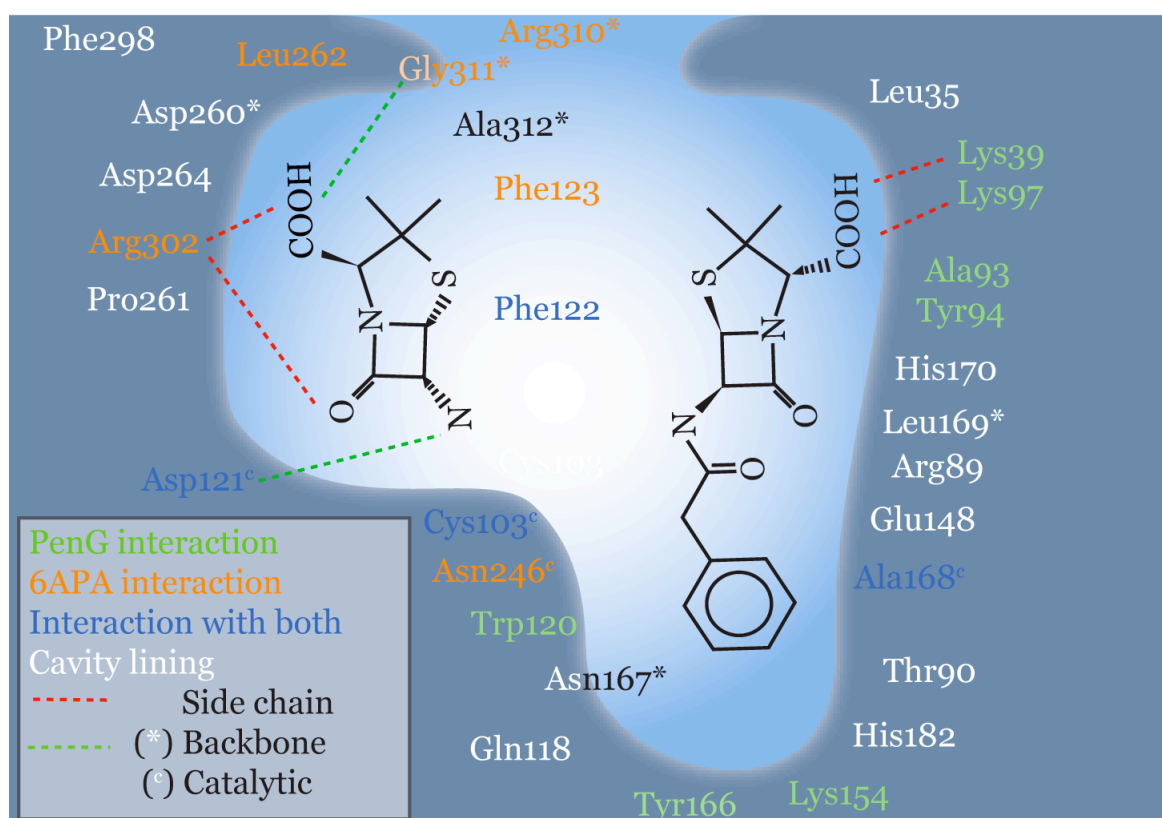


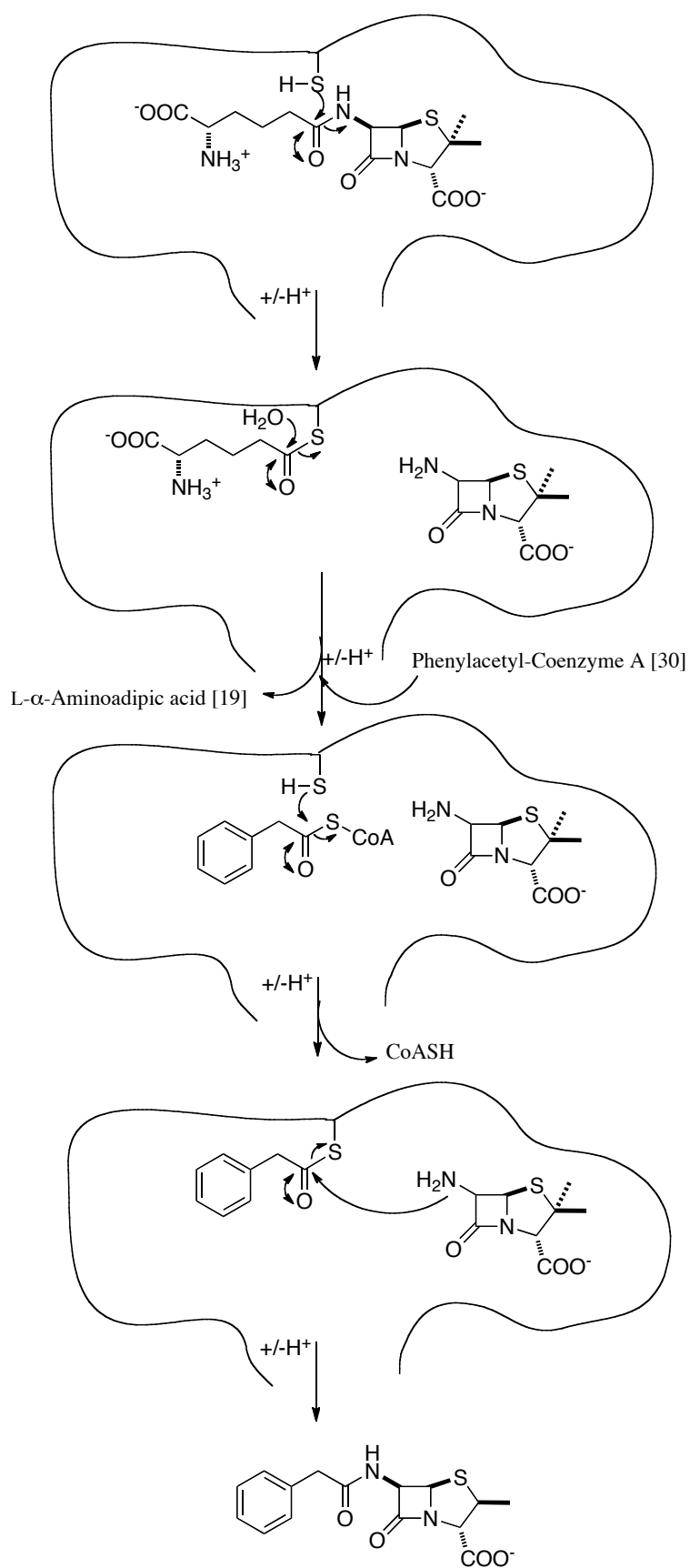
Figure 1.7 To show the AT storage pocket of AT (Image kindly provided by Marcel Bokhove).

It was proposed that once IPN [18] has bound to the active site of AT, nucleophilic attack occurs at the amide bond between the penicillin nucleus and the α -aminoadipic acid side chain. An α -aminoadipic acid-enzyme intermediate is formed and 6-APA [1] remains tightly bound in the penicillin nucleus binding pocket. Hydrolysis of the acyl-enzyme intermediate releases α -aminoadipic acid. Phenylacetyl-CoA binds to the side chain binding pocket and phenylacylation of the enzyme takes place. Nucleophilic

attack of the ester bond of the phenylacetyl-enzyme intermediate by 6-APA [1] completes the formation of Penicillin G [5] which is released from the enzyme (§ Scheme 1.5).

This mechanism was based on the kinetic information available at the time. Determination of the K_m value for the three main activities of AT has been achieved however, since Michaelis constants depend on assay conditions such as pH and ionic strength the data cannot be compared directly between sources. It is generally observed that AAT activity is much more efficient than IAT activity. When IPN [18] is hydrolysed 6-APA [1] is bound to the active site of AT until it is converted into Penicillin G [5] in the presence of phenylacetyl-CoA. The rate of this step slows down as the concentration of phenylacetyl-CoA [30] decreases, which suggests 6-APA [1] competitively inhibits the binding of IPN [18].

In summary, these findings particularly the broad substrate specificity of AT make it worthy of further investigation towards its potential exploitation for the improved production of β -lactam antibiotics.



Scheme 1.5 The proposed mechanism for IAT activity of AT (Hart 1996)

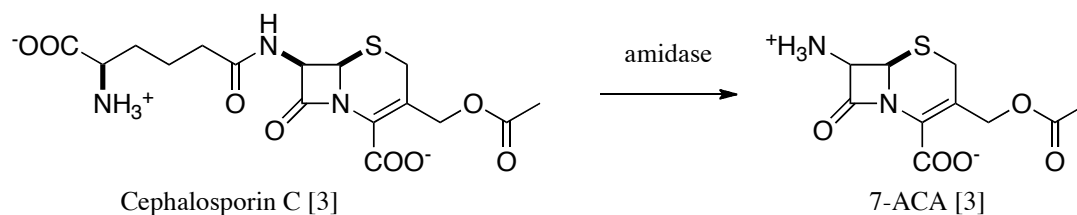
1.7 Project Aims

The improved production of β -lactam antibiotics in use today and the development of new analogues for which resistance is not (yet) an issue are major goals of the pharmaceutical industry. The aim of this project is to modify AT towards achieving these goals. As has been discussed AT has a broad substrate specificity, which could be exploited and broadened further using directed evolution to meet the demand for new manufacturing routes preferably based on fermentation of final or late-stage intermediates particularly towards the production of cephalosporins.

Although cephalosporins are generally regarded as better antibiotics due to their broader spectrum of activity and higher potency their use is limited by the cost and complexity involved in producing them. Production of novel β -lactams is semi-synthetic in that the nucleus, either 6-APA [1], 7-ADCA [11] or 7-ACA [4] is obtained by fermentation methods then acylated with a chemically synthesized side chain. For semi-synthetic cephalosporins, 7-ADCA [11] or 7-ACA [4] are required. However obtaining these cephalosporin nuclei is difficult.

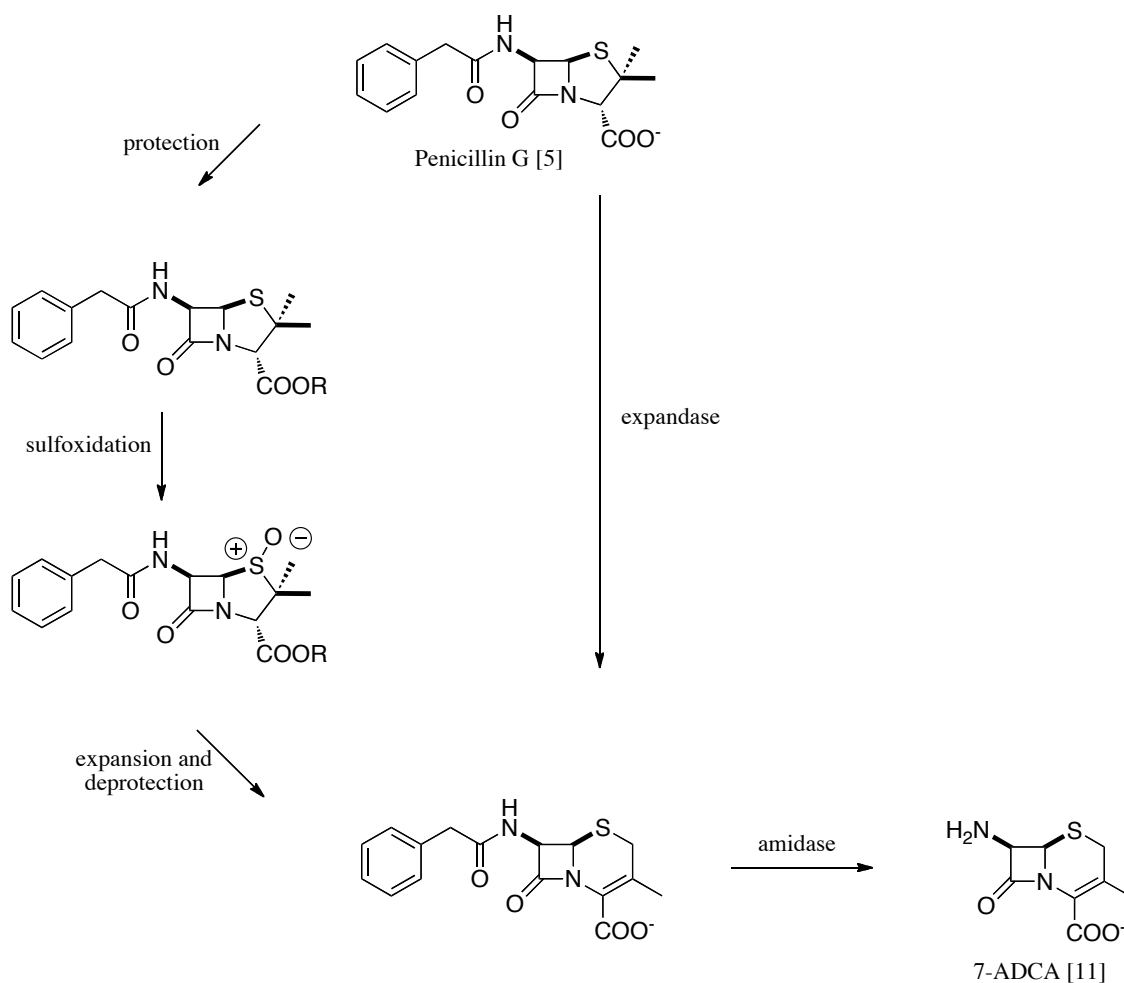
Current manufacturing processes are based on fermentation of natural β -lactams which are then extracted from the fermentation mixture and the side chains cleaved enzymatically to obtain the β -lactam nucleus. 6-APA [1] owes its ease of production to two advantages in Penicillin G [5]. Firstly, the hydrophobic phenylacetyl side chain allows Penicillin G [5] to be easily extracted from the fermentation mixture with organic solvent. Secondly, there are known acylases that are capable of cleaving this side chain thus giving 6-APA [1]. In the case of Cephalosporin C [3], the α -aminoadipoyl side chain does not permit easy extraction from the fermentation for subsequent side chain cleavage.

There are currently two routes towards the production of the cephalosporin nuclei 7-ACA [4] and 7-ADCA [11]. 7-ACA [4] can be obtained from the fermentation of high Cephalosporin C [3] producing strains. The resulting fermentation broth is purified and the side chain is cleaved from Cephalosporin C [3] using an amidase to give 7-ACA [4] which is isolated by precipitation. (Scheme 1.6). Amidases used for the cleavage of the side chain are not efficient and the yields of 7-ACA [4] obtained are low. Current work focuses on the development of efficient amidases.



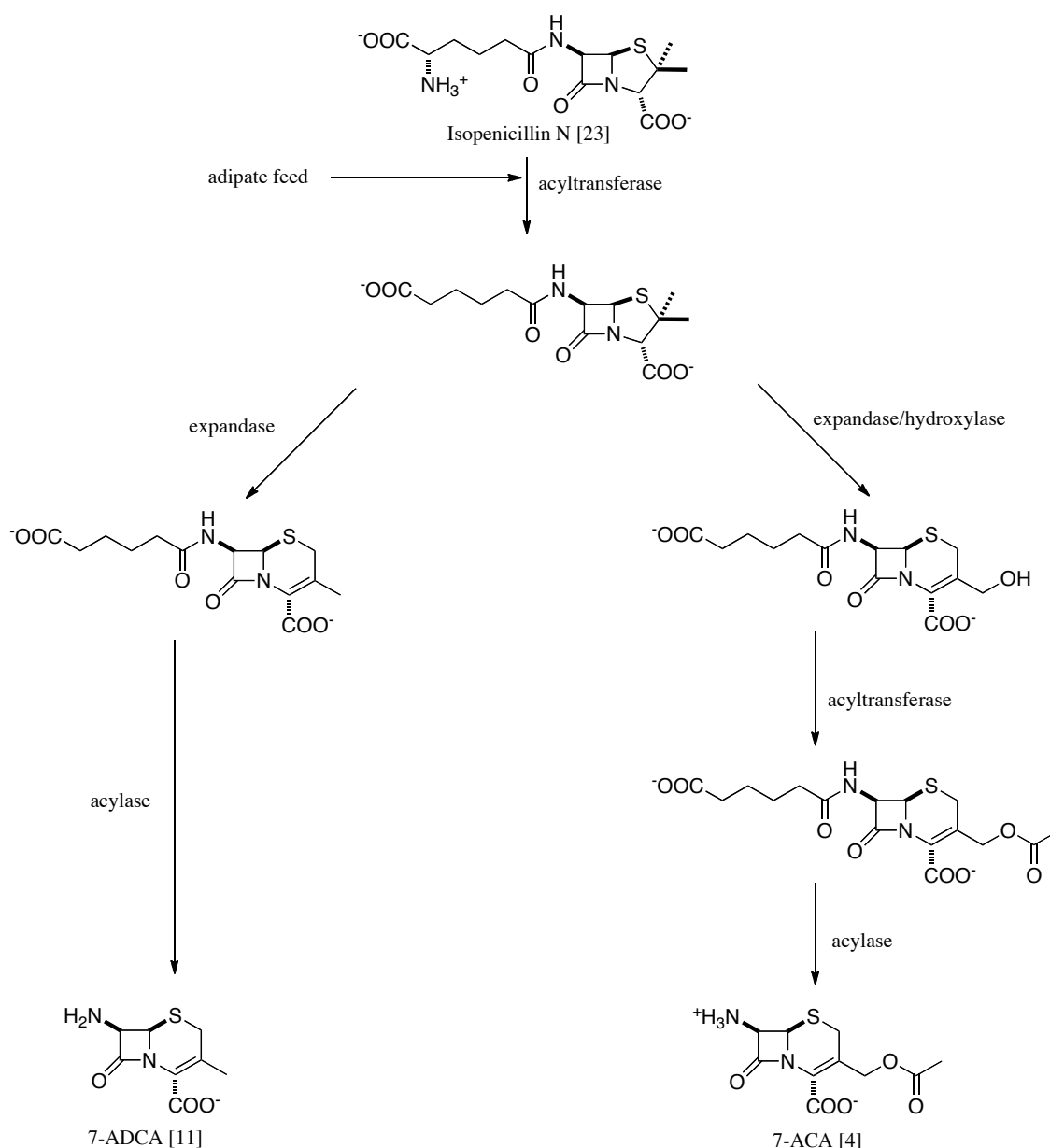
Scheme 1.6 Production of 7-ACA [4]

7-ADCA [11] can be obtained from either Penicillin G or Penicillin V. The penicillin is produced by fermentation and then the five-membered thiazolidine ring is expanded using either traditional chemical methods or increasingly, enzymatic methods from genetic engineering of the biosynthetic pathways. Enzymatic cleavage of the side chain results in 7-ADCA [11]. (Scheme 1.7).



Scheme 1.7 Production of 7-ADCA [11]

The method of chemical expansion is environmentally unfriendly therefore enzymatic methods have been developed. Key breakthroughs in the engineering of biosynthetic pathways towards production of cephalosporins include the production of deacetoxycephalosporin C in recombinant *P. chrysogenum* containing the *cefE* gene from *S. clavuligerus* resulting in the enzymatic expansion of Penicillin N [23] (Cantwell, Beckmann et al. 1990).



Scheme 1.8 The production of cephalosporin intermediates via production of adipoyl-cephalosporins in recombinant strains of *Penicillium chrysogenum* (Crawford, Stepan et al. 1995)

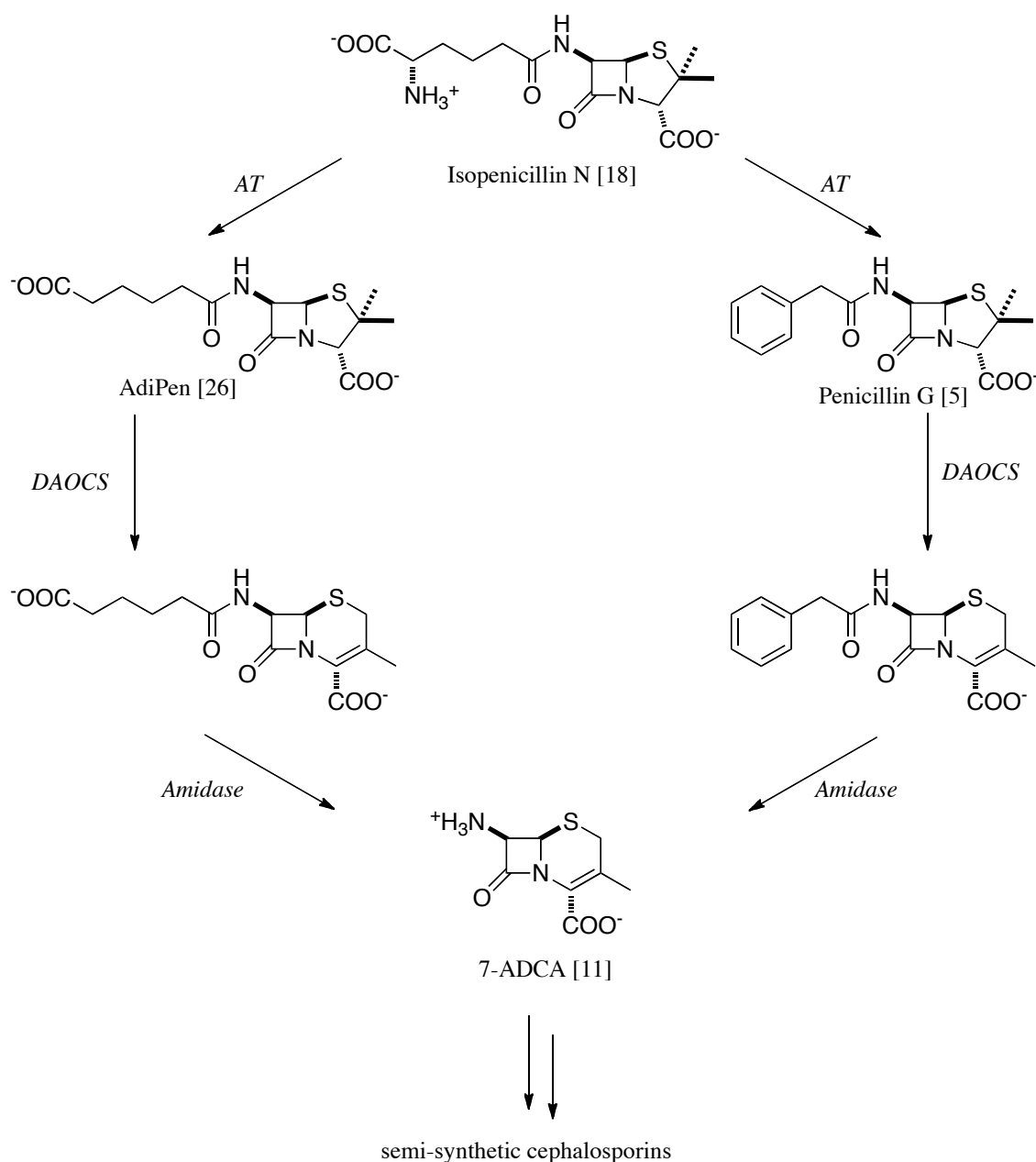
This idea was taken a step further and the use of *P. chrysogenum* strains containing either the expandase from *S. clavuligerus* or the expandase and hydroxylase from *C. acremonium* fed with adipic acid and subsequent cleavage of the fermentation product side chains gave 7-ADCA [11] and 7-ACA [4] respectively (Crawford, Stepan et al. 1995) (§ Scheme 1.8).

These approaches can be improved using strain improvement methods and directed evolution techniques to increase the efficiency of the fermentation processes and the subsequent amidase cleavage reactions. However, they do not get around the problem of extraction of the products from fermentation mixtures due to the presence of the aminoadipoyl and adipoyl side chains. Extraction could be improved by the presence of a hydrophobic side chain such as the phenylacetyl side chain in Penicillin G [5].

This makes AT an obvious choice for improving the production of 7-ADCA [11] and 7-ACA [4]. As well as improving its activity for the natural reaction and Penicillin G [5], its activity towards the adipoyl side chain towards the production of AdiPen [26] could also be improved. Both Penicillin G [5] and AdiPen [26] are substrates for DAOCS the resulting expanded products can then be cleaved to give 7-ADCA [11] as shown in Scheme 1.9.

There are advantages to both the routes shown in Scheme 1.9. In the first route, although the first transacylation step by AT is not as efficient as the natural reaction of AT, the resulting AdiPen [26] is a much better substrate for the expandase enzyme. In the case of route 2, the opposite is true; transacylation is efficient as it is the natural reaction of AT, however the subsequent ring expansion is not so easy.

The second route is more preferable due to the presence of the hydrophobic phenylacetyl side chain, which would allow for more efficient extraction of the Cephalosporin G [31] product from the fermentation mixture.



Scheme 1.9 2 routes to 7-ADCA [11] using AT

An even more ambitious approach would be to bypass both the transacylation of 6-APA [1] and subsequent ring expansion by using AT to directly transacylate Cephalosporin C [3] to produce Cephalosporin G [31], which could be easily extracted from fermentation mixtures and the side chain removed with an amidase to give 7-ADCA [11].

Although AT has demonstrated broad substrate specificity for the side chains of penicillins, this approach would require an AT variant with altered substrate specificity for the nucleus *i.e.* from penicillin nucleus 6-APA [1] to a cephalosporin nucleus, either 7-ACA [4] or 7-ADCA [11]. Unpublished work from the group has shown that AT has

low activity towards cephalosporin substrates (Tobin 1994) and a therefore more achievable goal of improving an existing activity rather than introducing a completely new one.

This thesis describes the search for AT mutants with improved IAH and AAT activities which when further combined would lead to an overall improvement in AT transacylase activity (IAT). Chapter 2 describes the use of an affinity screening method towards identifying AT mutants with improved AAT activity. Chapters 3 and 4 describe work towards developing a screening method for the identification of AT mutants with improved IAT activity.

CHAPTER TWO

Identification of AT Mutants with Improved AAT Activity

2.1 Introduction

Previous work in the group had concentrated on the acylation activity of AT towards various substrates with considerable success. The ultimate goal of the project is to modify AT with respect to cephalosporin substrates. It had been observed (Tobin, 1994) that AT has some activity, albeit low, with the cephalosporin nucleus, 7-ADCA [11]. So, instead of trying to introduce a new substrate specificity to AT ‘just’ an improvement on its natural activity was required. To this end work began with a rational approach to improving AT’s acylation activity and altering its substrate specificity.

2.2 A Rational Approach

In order to rationally identify the important residues of an enzyme, crystallographic data is needed, ideally a substrate bound co-crystal structure. Unfortunately, this data is not available for AT. This is due to the difficulty of obtaining pure homogenous preparations of AT, as the pure enzyme is subject to severe aggregation. However, this problem is overcome with the Cys103Ala mutant. Although this mutant does not have the ability to be processed into the mature heterodimeric protein and therefore corresponds to the proenzyme form of AT, it can be purified in large yields allowing some crystallographic data to be obtained (Hensgens, Kroezinga et al. 2002). This crystallographic data was used by the group to propose AT–IPN binding models, which led to the identification of key residues within the active site of AT that were subjected to further investigation.

2.2.1 Proposed Key AT Residues

A triad of residues, namely Arg89, Glu148 and Lys154, were believed to form part of a salt bridge with a carboxylate anion, initially that of IPN [18], later with that of 6-APA [1].

Hydrophobic interactions were thought possible between residues Thr90, Ala93, Phe122, Tyr166, and Ala168. An edge-to-face interaction between Phe122 and Phe123 was also believed to exist.

Residues that were believed to be in and around the active site included, Arg89, Thr90, Glu91 and Ala93 of the α -subunit, and Cys103, Trp120, Phe123, Glu148, Lys154,

Tyr166, Ala168 and Ser181 of the β -subunit. Residues Lys97 and His170 were also considered important due to their close proximity to the active site.

A number of the residues, Leu35, Thr90, Tyr94, Ala93, Lys97, Phe122, Phe123, Tyr166, Ala168 and His170 were subjected to mutagenesis experiments, expressed and characterised by SDS-PAGE and their activities compared to that of wild type AT for various conversions as follows.

2.2.2 Site Directed Mutagenesis of Key AT Residues

The first residues to be investigated were Thr90 and Ala168 (Derome 2003) which were believed to be in the active site of AT. It was rationalised that more room could be created in the active site of AT to accommodate larger cephalosporin nuclei by removing the methyl group from these residues by switching threonine for serine, and alanine for glycine. It was anticipated that this would create enough space to alter substrate specificity while at the same time not alter main chain functions. These mutants were made using site directed mutagenesis. The resultant proteins were analysed by SDS-PAGE and seen to fold correctly. The activity of the mutants, Thr90Ser and Ala168Gly, was compared to wild type AT by bioplate assay. For the conversion of 6-APA [1] with PACoA [30] into Penicillin G [5], Thr90Ser had similar activity to wild type AT. However, Ala168Gly had a much-reduced activity, which showed that it was indeed an important residue for AT activity. For the conversion of *O*-carbamoyl-7-aminodeacetylcephalosporanic acid, OC-7-ADCA [29] with PACoA [30] into *O*-carbamoyldeacetylcephalosporin G, OCDAG [24], Ala168Gly again showed a decrease in activity compared to wild type AT. However, Thr90Ser showed an increase of 5%. These results validated the approach and other residues were investigated.

Following on from the success of mutant Thr90Ser, Tyr166 was investigated (Cheung 2006). It is directly opposite Thr90 in the active site so the same strategy was applied, the creation of space by removing a functional group from the residue, in this case removal of the hydroxyl group by substituting tyrosine for phenylalanine. This mutant did indeed show improved activity for OC-7-ADCA [29] and so the double mutant Thr90Ser Tyr166Phe was made with the aim of opening up the active site even more.

However, this proved a step too far as although this mutant did express correctly (as seen by SDS-PAGE), it showed no improvement in activity for OC-7-ADCA [29].

The edge-to-face interaction between Phe122 and Phe123 was also investigated with a series of site directed mutagenesis experiments (Cheung 2006). It was considered important to conserve the hydrophobicity at these positions so substitutions were made for other hydrophobic residues, valine, leucine, isoleucine and methionine. Proline was considered to be potentially too disruptive to the protein structure. SDS-PAGE analysis revealed that substitution was not tolerated at position 122 as these proteins did not fold properly. Although substitutions were tolerated at position 123 in terms of folding, none of the mutants showed any improvement in activity.

Attention was then turned to residues in the outer layer of the active site. At this time it was believed that the triad of residues, namely Arg89, Glu148 and Lys154, formed a salt bridge with the carboxylate anion of 6-APA [1], and not that of IPN [18] as originally supposed. This led to the identification of other residues deemed worthy of investigation; Leu35, Tyr94, Lys97 and His170.

Leu35 is located above the carboxylate anion of the IPN [18] side chain. Substitutions at Leu35 were made to retain the hydrophobicity (valine, leucine, isoleucine and methionine). Not all the substitutions were tolerated in terms of protein structure. However, of the mutants that did fold correctly, one, Leu35Met, did show some improvement in activity towards OCDAG [24] formation (Cheung 2006).

Tyr94 points towards to the binding site of the enzyme. The mutant Tyr94Phe was made in an attempt to again create more space in the active site. Although there was no great improvement in activity, there was some activity despite partial proteolysis of the expressed protein (as seen by SDS-PAGE), which suggests that the hydroxyl group at this position was not essential (Cheung 2006).

Lys97 was investigated due to its close proximity to Cys103 (Khara 2007). Site saturation mutagenesis was carried out at this residue, which led to the identification of two interesting mutants and the suggestion that Lys97 was a 'hotspot'. Lys97Arg showed an increase in activity when compared to wild type AT for the conversion of 6-APA [1] into Penicillin G [5]. Lys97Val showed an increased conversion of OC-7-ADCA [29] into OCDAG [24]. Interestingly, the amino acid sequences for the *penDE*

gene from *P. chrysogenum* and *A. nidulans* differ in the same way at this point, *i.e.* a lysine residue at position 97 in *P. chrysogenum* and valine in *A. nidulans*. This led to a comparison of the genes from both organisms in the hope that by going from *P. chrysogenum* to *A. nidulans* cephalosporin activity would be favoured (§ Appendix E amino acid sequences for both organisms).

The following mutants were made accordingly, Ala98Glu and Gln172His and investigated (Cheung 2006). Both mutants showed an improvement in conversion of OC-7-ADCA [29] for the formation of OCDAG [24], unfortunately the double mutant, Ala98Glu Gln172His, did not.

In summary, 5 useful mutants had been identified by the group, using a rational approach based on a putative binding model: Thr90Ser, Tyr166Phe, Leu35Met, Ala98Glu and Gln172His. However, this number was too few for a round of DNA shuffling and in the absence of any new crystallographic data a random approach was needed in order to identify useful AT mutants. As mutations at positions other than the active site and often far from, the active site can have a beneficial effect on enzyme activity but are impossible to predict, a random approach was employed.

2.3 A Random Approach

Random libraries were created *via* error prone PCR (epPCR) using Stratagene's GeneMorph II Random Mutagenesis kit and methods to screen the libraries developed.

Bioplate assay is really only suitable for analysing a handful of mutants at a time. A screening or selection method was needed that would allow thousands of mutants to be analysed rapidly. The use of an *E. coli* strain (*E. coli* ESS) that was sensitive to varying amounts of antibiotic was key to the methods developed in the group.

2.3.1 High-Throughput Liquid Assay Screening and Results

Initially a high throughput method was developed (Derome 2003). This involved the use of a robotic colony picker to inoculate a 96-well micro titre plate (MTP). Induction with IPTG followed by lysis with a commercially available reagent (BPER II) gave cell free extracts to which potassium clavulanate was added to inactivate any β -lactamase present, which would have interfered with the screen. Substrates (OC-7-ADCA [29] and PACoA [30]) were added and the reactions incubated. Aliquots of the resulting

mixture were mixed with liquid media and added to a replica plate containing *E. coli* ESS. Measuring the OD₆₀₀ of the resulting solutions after a period of incubation allowed the amount of conversion to be determined by reference to an internal standard and the mutant responsible could be selected from the master plate for further analysis.

A screen was carried out using this method albeit it smaller than originally planned due to the prohibitive cost of PACoA [30] - only 3000 mutants as opposed to the desired 10000. Although some potential 'hits' were isolated, upon further analysis these turned out to be false positives. This was probably due to the fact that the library screened had only a low mutation rate (<50% of the clones contained mutation/s) making it difficult to pick out any useful mutants from such a high background of wild type AT especially when the number of colonies actually screened was smaller than desired.

Although initially promising this screening method gave rise to a number of issues, which needed to be addressed if useful AT mutants were to be identified. Firstly, library quality; a higher mutation library was needed. Secondly, the prohibitive cost of PACoA [30] was limiting the size of screen that could be carried out. Thirdly, the equipment used to carry out the screen was only available at our funding partners (DSM) laboratory in The Netherlands, a method that could be carried out in Manchester was highly desirable.

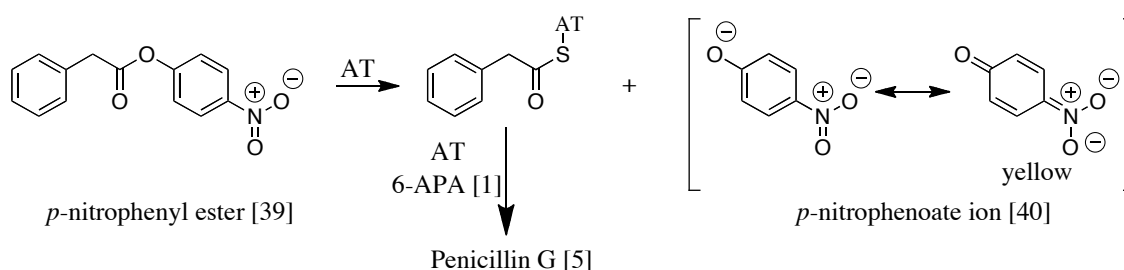
2.3.2 Library Improvement

The problem of library quality was solved by altering the parameters used during library creation with Stratagene's GeneMorph II Random Mutagenesis kit, which led to the creation of a high mutation library (90% of the clones contained mutation/s) (Khara 2007).

2.3.3 Alternative Acyl Donor Substrates

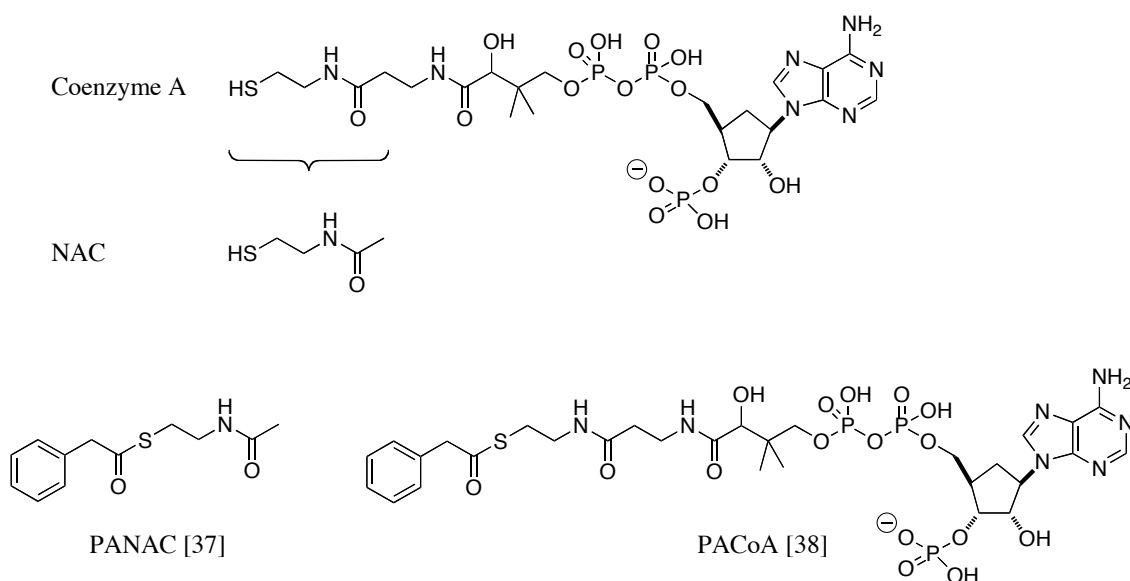
To address the issue of substrate cost, alternative substrates/analogues of PACoA [30] were sought. Initially, a *para*-nitrophenyl ester [39] was considered with the possibility of exploiting the *para*-nitrophenolate ion [40] in a colorimetric assay (Figure 2.1). However, experimental determination of the rates of AT catalysed and uncatalysed hydrolysis of the *para*-nitrophenyl ester [39] found them to be too similar and the ester was deemed too hydrolytically labile to be useful. Substituting the ester bond for the

stronger amide analogue would have reduced the rate of hydrolysis. However, it would also have been structurally quite different from PACoA [30] and during the screening process would possibly lead to the selection of mutants that were not selective for PACoA [38] (Khara 2007).



Scheme 2.1 To show the formation of the *para*-nitrophenoate ion [40]

Another alternative was sought and found with the use of *N*-acetyl cysteamine. When considered more closely, the structure of PACoA [30] is a thioester made up of two parts; the acyl group carrier, coenzyme A, and the phenyl acyl component, which is transferred during acylation to the penicillin nucleus, 6-APA [1]. By using the thiol, *N*-acetyl cysteamine, an analogous thioester acyl donor (PANAC [37]) was made which was a much cheaper, coenzyme A alternative. This substrate was found to be utilised by AT and although it was not quite as good a substrate as the natural substrate, PACoA [30], it was good enough for screening purposes. The only drawback was the possibility of obtaining ‘NAC’ selective mutants from the screen. However, this was easily solved; any mutants found from screens using PANAC with improved activity for the formation of penicillin G [5] were verified using PACoA [30]. PANAC [37] is also more hydrophobic than PACoA [30] so 5% THF was required to dissolve it. However, the presence of organic solvent was found not to be detrimental to AT activity (Cheung 2006).



Scheme 2.2 To compare Coenzyme A with *N*-acetylcysteamine

2.3.4 Medium Throughput Amylose Affinity Membrane Screen

Another screening method was then developed by previous group members (Cheung 2006; Khara 2007) which avoided the use of robotic equipment that was not available to the group in Manchester. This time, a medium throughput method, which utilised an amylose affinity membrane (Cattoli and Sarti 2002) that AT-MBP fusion proteins could bind to was employed. The screening procedure was also used by the author and is detailed below and shown in Figure 2.1. (For more experimental detail see § 6.5.1).

E. coli colonies containing the AT clones are grown on agar plates supplemented with chloramphenicol ($30 \mu\text{g mL}^{-1}$) and IPTG (0.3 mM). The colonies are then ‘lifted’ using an amylose affinity membrane. Whilst on the membrane the cells are lysed to release the AT-MBP fusion protein, which binds to the membrane *via* MBP-amylose affinity, exposing AT on the surface of the membrane. In the meantime, the original agar ‘master’ plate is incubated further to regenerate the colonies then stored until required at 4°C .

Substrates, i.e. penicillin or cephalosporin nucleus plus acyl donor, can be sprayed onto the membrane at this point and allowed to react, catalysed by the AT present on the membrane. The membrane is then placed face down onto an agar plate containing the super sensitive *E. coli* strain, *E. coli* ESS, and the whole assembly incubated. Alternatively, substrates can be added directly to the *E. coli* ESS-agar plate. Kill zones

in the *E. coli* ESS-agar plate, observed as clear halos, correspond to the formation of antibiotic, with larger kill zones indicating greater AT acylation activity. The colony responsible can then be selected from the ‘master’ plate for further investigation.

For screen development and initial screening the natural AT conversion of 6-APA [1] to penicillin G [5] formation was used. As already hinted, the method of substrate application in the screen depended on the solubility of the acyl donor; less soluble substrates were sprayed as aqueous solutions containing 5% THF onto the membrane, more soluble substrates were added to the agar during *E. coli* ESS-agar plate preparation.

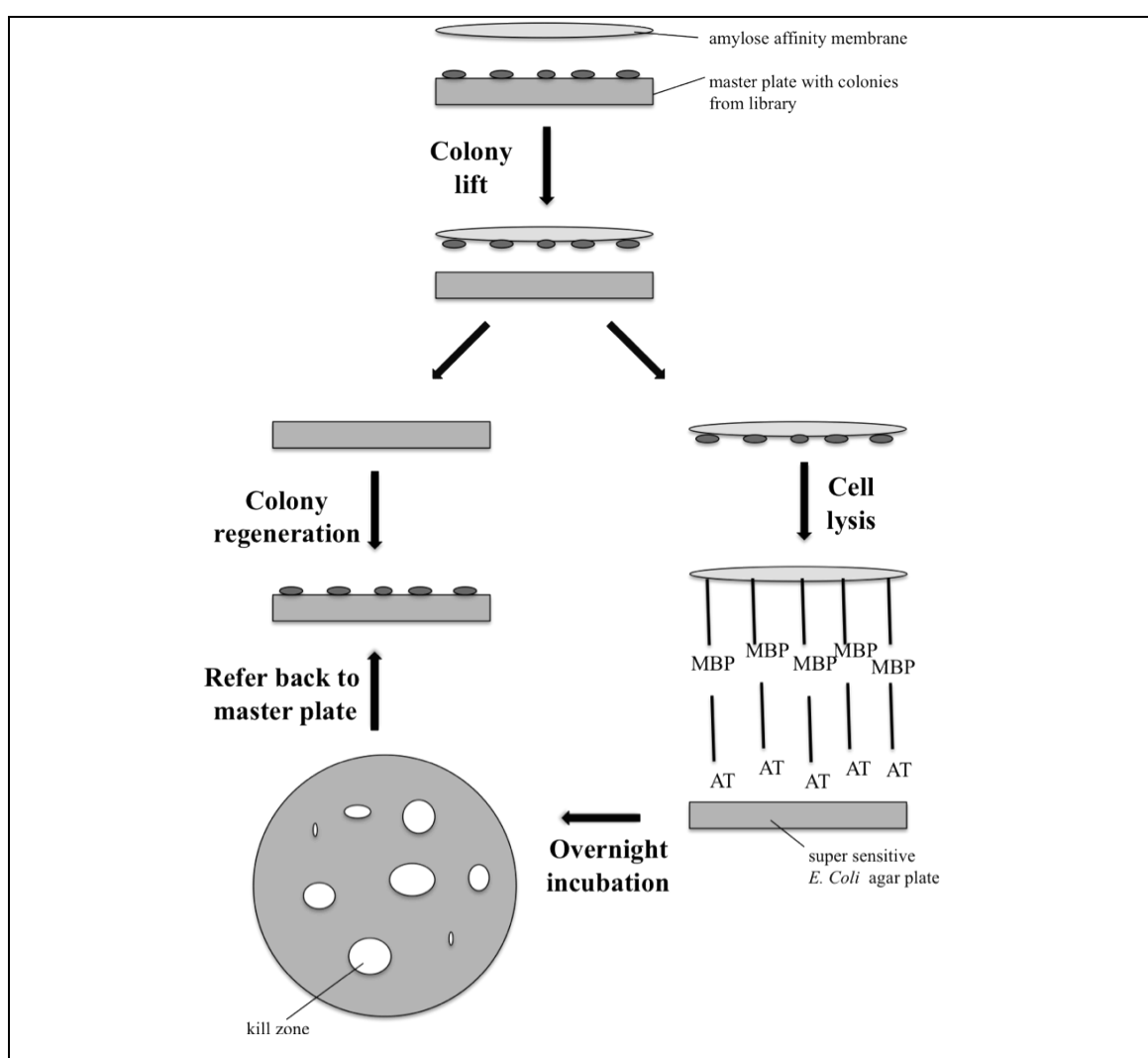


Figure 2.1 A diagram to show the amylose affinity membrane screen

This screening procedure was used to screen the library with great success and a number of useful AT acylation mutants were identified. In total, approximately 50,000 clones were rapidly screened, which led to the identification of 7 new residues of interest that could not have been predicted from crystal structure data due to their distant locations from the active site of AT (Khara 2007).

These residues were recombined in various permutations and the resulting recombinants' activity determined for the acylation of 6-APA with AdiNAC [33], PANAC [37] and PACoA [30] (Khara 2007). In the case of the adipoyl side chain, only mutants with good activity with AdiNAC [33] were investigated with AdiCoA [32] due to the expense of the substrate. Recombination of the mutations generally had a cumulative effect on activity. Particularly noteworthy was the recombinant Asp34Asn Ala106Val His182Asn His219Tyr. This was found to have a 30 fold improvement on wild type activity for the formation of AdiPen [26] from 6-APA [1] and AdiCoA [32] (18 fold improvement when AdiNAC [33] was used).

A summary of the recombinants and their activities is shown in Table 2.1. Four crosses represents wild type activity, minus represents a dramatic loss in the substrate activity. Comparisons can only be made within, but not between columns as PACoA [30] is a much better co-substrate than PANAC [37] or AdiNAC [33], and AdiCoA [32] is a better co-substrate than AdiNAC [33].

Table 2.1 AT Recombinants with improved acylation activity (Khara 2007)

Amino Acid Change	PACoA	PANAC	AdiNAC	AdiCoA
wt	++++	++++	++++	++++
Single Mutations				
D34N	++++	+++ (+)	+++ (+)	ND
K64E	++++ (+)	++++ (+)	++++	ND
K97R*	++++ (+)	++++ (+)	++++ (+)	+++++
A106V	+++++ (+)	++++ (+)	+++++	+++++ +++
H182N	+ (+)	++ (+)	+++++ +	+++++ +++ (+)
H219Y	+++++	++++ (+)	++++ (+)	++++ (+)
N239K	+++++	++++ (+)	++	ND
G278R	+++++	++++ (+)	++++ (+)	ND
Double Recombinants				
K97R H219Y	+++++ +	+++++ +	+++++	ND
K97R N239K	+++++ (+)	++++ (+)	++++	ND
H219 N239K	+++++ +	++++ (+)	++++	++++ (+)
D34N A106V	+++++ +	++++ (+)	++++ (+)	+++++ +++++
K39E K64E	+	+++++	++++ (+)	ND
A106V H182N	+++	+++ (+)	+++++ (+)	+++++ + (+)
K39E H182N	(+)	+	+++++ (+)	ND

H182N H219Y	+	(+)	+++++ +	ND
K39E A106V	+(+)	+++++ +	+++++	ND
A106V H219Y	+++++ +	+++++ (+)	+++++ (+)	+++++ ++(+)
K39E H219Y	+	+++++	+++++ (+)	ND
Triple Recombinants				
K97R H219Y N239K	+++++ ++	+++++ +(+)	+++++ (+)	+++++ (+)
K39E A106V H182N	(+)	++	+++++ ++	ND
K39E H182N H219Y	(+)	+(+)	+++++ ++	ND
K39E A106V H219Y	+(+)	+++++ (+)	+++++ +(+)	ND
A106V H182N H219Y	+++(+)	+++(+)	+++++ ++(+)	+++++ +++++ (+)
A106V H219Y N239K	+++++ (+)	+++++ (+)	+++++ (+)	+++++ +++++ (+)
Quadruple Recombinants				
D34N A106V H182N H219Y	+++++	+++++ (+)	+++++ +++++	+++++ +++++ +++++ (+)
K97R A106V H219Y N239K	+++++ ++	+++++ +(+)	+++++ (+)	+++++ +++
Quintuple Recombinant				
D34N K97R A106V H219Y N239K	+++++ ++(+)	+++++	+++++	+++++ +++++

ND = *not determined*.

**Rational mutant*

2.4 Further Library Screening

The author continued screening the high mutation library using the amylose affinity membrane screen, for mutants with improved activity, with respect to wild type AT, for the formation of AdiPen [26] from 6-APA [1] and AdiNAC [33].

In total approximately 10,000 clones were screened. This led to the initial identification of 60 colonies that had increased activity with respect to wild type AT. In order to verify this result and narrow down these possibilities further, the colonies were tooth picked onto an agar plate in a grid to give a new master plate and re-assayed using the amylose affinity screen. This resulted in a final six candidates that showed the most improvement in activity when compared to wild type AT.

DNA sequencing revealed three of these to be wild type, two contained the single mutation, Gly278Arg, which had been found previously by former group member, Khara (Khara 2007). The final variant was the double mutant, Arg70Leu Asn267Ser, not found in previous screens.

Obtaining wild type AT from the screen can be explained when colony size is considered. In the initial screen the colonies would not have all been the same size due to various growth factors. It was also impossible during the tooth picking process to ensure a uniform colony size. Larger colonies will give larger kill zones due to the increased amount of AT present and not necessarily because the AT present is any more active than wild type. It was quite difficult in practice to take into account the relative size of the colony, compared to the size of kill zone it subsequently produced. This perhaps goes towards explaining why of the clones selected from the screen, 50% were wild type.

The reoccurrence between screens of the single mutant Gly278Arg implies that the method used to sample the library is sufficient. It is likely that the library had been thoroughly screened at this point and most of the mutants contained in the library with improved activity for the acylation of 6-APA [1] with various side chains identified.

This leaves the double mutant Arg70Leu Asn267Ser, which was investigated further as follows. As well as comparing with wild type AT, it was decided to compare the mutants obtained from the screen with Asp34Asn Ala106Val. This double mutant had

been obtained from a screen by previous group member Khara (Khara 2007) and was found to have a considerably greater activity than wild type AT for the acylation of 6-APA [1] with both the phenyl acyl (PANAC [37] and PACoA [30]) and adipoyl side chains (ADiNAC [33] and AdiCoA [32]) (§ Table 2.1).

The proteins were expressed and purified then characterised by SDS-PAGE to ensure post-translational cleavage had occurred. Initial kinetic studies (§ 6.6) were carried out for the conversion of AdiPen [26] from 6-APA [1] and ADiNAC [33]. The data obtained showed that Arg70Leu Asn267Ser did have improved activity when compared with wild type AT although it was not quite as improved as that of Asp34Asn Ala106Val (Figure 2.2). However, at this stage it was not known whether both the mutations, Arg70Leu and Asn267Ser, contributed equally to the observed activity of Arg70Leu Asn267Ser. It was possible that either was much superior to the other or that one had no contribution towards the observed activity or indeed, had a deleterious effect on the overall activity. It was therefore desirable to separate the double mutant into its constituent single mutants to determine their individual effect on activity.

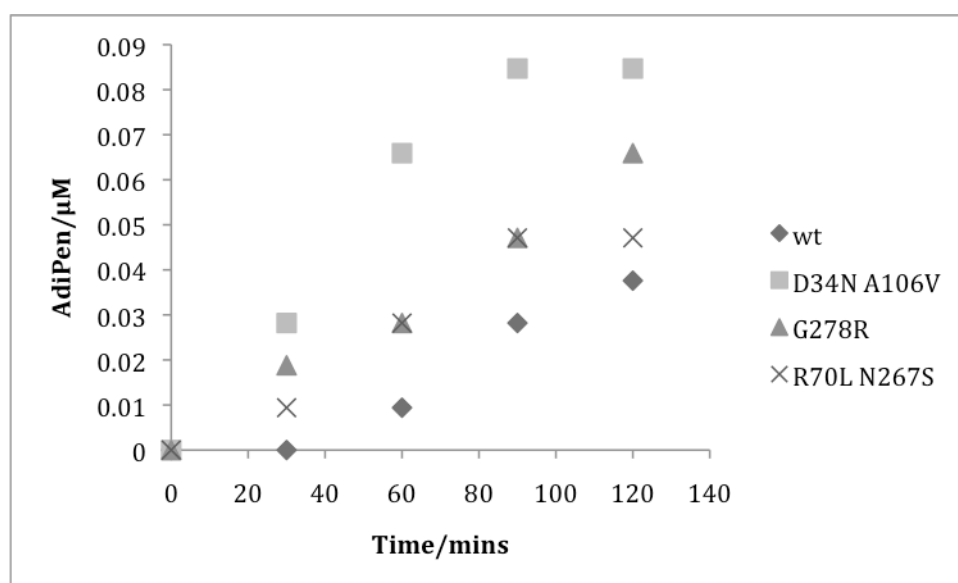


Figure 2.2 A graph to show the rate of formation of AdiPen [26] for various AT mutants

Site directed mutagenesis was used to obtain the Arg70Leu and Asn267Ser as single mutations for further kinetic studies. The proteins were expressed, purified then characterised by SDS-PAGE as before. Kinetic studies were carried out as before for the conversion of AdiPen [26] from 6-APA [1] and ADiNAC [33]. It was found that the

mutations individually did not contribute equally to the activity that had been observed for the double mutant. In fact, Arg70Leu had a decrease in activity when compared to wild type AT. However, Asn267Ser was found to have a much improved activity when compared with both the double mutant Arg70Leu Asn267Ser, and wild type AT and its activity was comparable to that of Asp34Asn Ala106Val (data not shown). To summarise, the activity of the various mutants went in the order:

Arg70Leu < wild type < Arg70Leu Asn267Ser < Asn267Ser = Asp34Asn Ala106Val

The fact that the activity of Arg70Leu had a lower activity than wild type and Asn267Ser was much higher was interesting but perhaps not so surprising. It was speculated that as AT uses coenzyme A derivatives as substrates, the presence of an AT variant with improved activity in *E. coli* could have a detrimental effect on cell growth, perhaps by hydrolysing any coenzyme A derivatives required by *E. coli* for growth. If this were the case it would have implications for the screening process.

Colonies containing AT mutants with increased activity would display less growth, therefore less AT would be present, resulting in a smaller kill zone, which would not lead to selection of the mutant from the screen. However, the presence of a weaker mutation allows stronger mutants to be found as the weaker mutant enables the colony to grow as normal, therefore compensating for the stronger mutants lack of growth thus leading to selection of the desired stronger mutant from the screen. In this case, Arg70Leu as the weaker mutant allowed colony growth ensuring the selection of the stronger Asn267Ser mutant from the screen.

Potentially, this means that mutants with increased activity would not be selected from the screen unless they are coupled with a less active mutant. A possible solution to this problem would be to add IPTG after a period of colony growth before cell lysis thus ensuring uniform colony growth and a fairer comparison of the resultant kill zones. During the employed screening process, the inducer, IPTG, was present in the agar plates from the beginning of colony growth.

It was also observed that protein expression levels for variants were generally lower than those of wild type. When expressing AT, the *E. coli* cell cultures undergo a growth period before expression is induced with the addition of IPTG. In principle, no AT should be present during this growth period. However, AT expression is under the

control of the *lac* operon which is known to be ‘leaky’, that is to say some protein is expressed before induction. Protein expression can be more tightly regulated with the use of the *lacI^q* gene, a mutated version of the *lacI* gene that leads to increased levels of the lac repressor. Although pHAR10a does have the *lacI^q* gene, there were doubts as to its integrity; it was believed there was a deletion in the gene (Shorrock 1998) and some even doubted its presence at all due to the derivation of the pHAR10a plasmid from the pUC series which contain truncated versions of the *lacI^q* gene (Derome 2003). However, this can be compensated for by using a host strain that does contain the *lacI^q* gene, like XL1-Blue. However, even with the use of the *lacI^q* gene, protein expression is not completely repressed; there is at least 30% AT expression without IPTG (Hart 1996; Shorrock 1998).

As the results of the kinetic studies showed an improvement in acylation with Asn267Ser it was decided to investigate the effect of the mutation further by combining with the individual mutations, Asp34Asn and Ala106Val, in various permutations in order to determine whether the improvement in activity observed for Asn267Ser would be additive as had been previously found for other mutations (Khara 2007).

The following mutants were made accordingly by site directed mutagenesis: the double mutants, Asp34Asn Asn267Ser and Ala106Val Asn267Ser and the triple mutant Asp34Asn Ala106Val Asn267Ser. The proteins were expressed and purified then characterised by SDS-PAGE as before.

Kinetic studies were again carried out as before for the conversion of AdiPen [26] from 6-APA [1] and AdiNAC [33] to determine the effect of the Asn267Ser mutation. When combined with Asp34Asn there was no improvement in activity on Asp34Asn alone and the double mutant Asp34Asn Asn267Ser was only comparable to wild type activity. When combined with Ala106Val there was a big improvement, the activity was greater than that observed for Asn267Ser alone and the double Asp34Asn Ala106Val. In the case of the triple mutant, Asp34Asn Ala106Val Asn267Ser, there was the greatest improvement in activity (§ Figure 2.3).

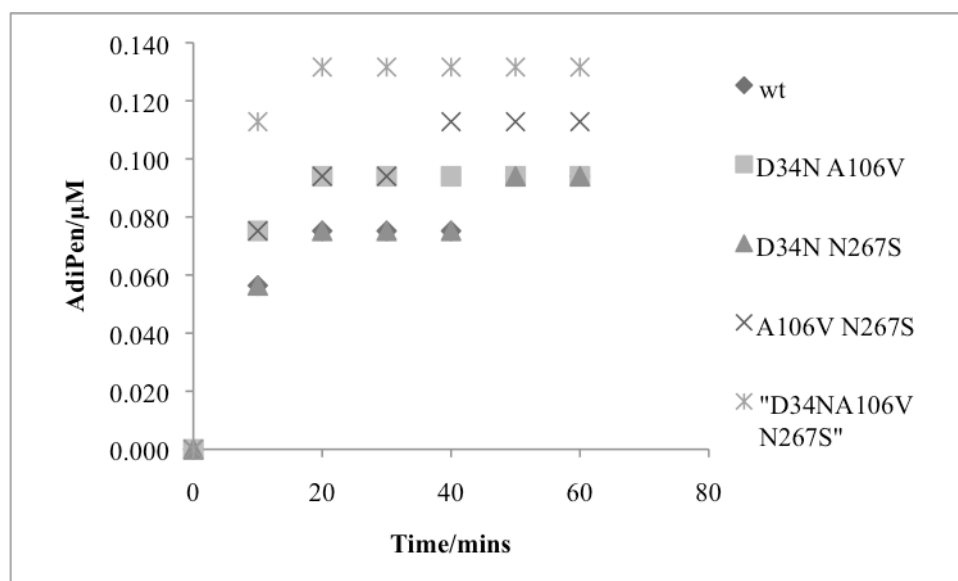


Figure 2.3 A graph to show the rate of formation of AdiPen [26] for various AT mutants

Overall the results showed that the inclusion of Asn267Ser did indeed have an additive effect on activity when combined with other mutations, except when combined with Asp34Asn. However, there was no detrimental effect on activity when Asn267Ser was combined with Asp34Asn. These results were reproduced when AdiCoA [32] was used as the acyl donor (data not shown). To summarise, the activity of the various mutants was of the following order:

wild type = Asp34Asn = Asp34Asn Asn267Ser < Asn267Ser = Asp34Asn Ala106Val < Ala106Val Asn267Ser < Asp34Asn Ala106Val Asn267Ser

When the amino acid sequences for the *penDE* gene from *P. chrysogenum* and *A. nidulans* were compared it was found that the sequences differed in the same way at this point with asparagine at position 267 in *P. chrysogenum* and serine in *A. nidulans* which further corroborates the suggestion that the substrate specificities for AT from each organism may differ (§ 2.2.2).

2.5 Chapter 2 Summary

Screening of the high mutation library using the amylose affinity membrane procedure led to the selection of the double mutant Arg70Leu Asn267Ser. Upon further investigation although the individual mutation Arg70Leu had decreased activity for the formation of AdiPen [26] from 6-APA [1] and AdiNAC [33], the Asn267Ser single

showed a large improvement on wild type activity. Recombination led to the formation of the triple mutant Asp34Asn Ala106Val Asn267Ser, which showed an even greater improvement for the acylation of 6-APA [1].

Selection of the previously identified Gly278Arg mutant suggested the library had been sufficiently screened for acylation of 6-APA [1]. Attention is now turned to using the amylose affinity membrane screen in order to identify beneficial mutants for the acylation of cephalosporin substrates.

CHAPTER THREE

Periplasmic Expression of AT as an Approach Towards Hydrolysis Screening

3.1 Introduction

Since a number of AT mutants with improved acylation activity had been identified, attention was turned to identifying AT mutants with improved hydrolysis activity. In order to do this a selection or screening method was needed. Unfortunately, the amylose affinity screening method was not suitable for this purpose.

The amylose affinity screening method takes advantage of the fact that during the course of the acylation reaction, 6-APA [1] is converted into its antibiotic, acylated form. However, during the course of hydrolysis the opposite occurs: the bioactive IPN [18] is converted into the less bioactive substrate, 6-APA [1]. If the amylose affinity screening method were used to find mutants with improved hydrolysis activity, the *E. coli* ESS would be killed at the beginning of the screen and therefore would not indicate hydrolysis. Retrospective application of *E. coli* ESS during the screening process once the hydrolysis had been allowed to take place was considered. However, in practice this was not feasible therefore another approach was needed.

When considering the hydrolysis step independently from the acylation step there is one major issue that needs to be addressed by any screening or selection method. When IPN [18] is hydrolysed, 6-APA [1] is formed which remains in the active site of AT. In normal circumstances 6-APA [1] is re-acylated by AT. However, if no acylation takes place, as would be the case for any hydrolysis screening or selection method, 6-APA [1] will remain in the active site of AT as 6-APA [1] has a high affinity for AT. 6-APA [1] blocks the active site of AT to further molecules of IPN [18] therefore effectively acting as an inhibitor of AT hydrolysis. A screening or selection method for the identification of hydrolysis mutants needs to overcome this problem. In principle there are two main ways this problem could be addressed. One approach would be to have a highly sensitive screening or selection method, which would be able to detect the small concentrations of IPN [18] and 6-APA [1] involved. Another approach would be to somehow remove 6-APA [1] from the active site.

3.1.1 The Periplasmic Expression of AT

One way to perhaps increase the sensitivity of any assay would be to increase the amount of AT present. Although each molecule of AT would perhaps only hydrolyse one molecule of IPN [18] if there was lots of AT present the overall amount of IPN [18]

hydrolysed to 6-APA [1] would be greater which would be easier to detect. In order to increase the amount of AT available the expression of AT in the periplasm of *E. coli* was considered.

The periplasm is the region of space between the inner and outer membranes of gram-negative bacteria such as *E. coli* and could be considered as the first line of defence of bacteria towards antibiotics. While AT is located in the cytoplasm of *E. coli*, IPN [18] can cross the outer membrane into the periplasmic space where it can inhibit cell wall formation thus killing the cell. However, if AT was also present in the periplasm it could hydrolyse and therefore inactivate the IPN [18] present allowing survival of the cell. This situation would be comparable to the presence of β -lactamases in bacteria, which also operate in the periplasm and protect the organism by hydrolysing the β -lactam ring of β -lactam antibiotics (§ 1.4.1). Plating the library on IPN [18] concentrations at MIC or higher would enable life/death selection; mutants capable of destroying IPN [18] due to their increased hydrolytic capability would result in the survival of the corresponding colony. Selection of the colony would lead to identification of the responsible mutant from the library. Wild type AT or mutants with wild type activity or lower would not be able to inactivate IPN [18] by hydrolysis and therefore would not survive.

3.1.2 Targeting Proteins to the Periplasm

In 1999, Günter Blobel was awarded a Nobel Prize for recognising that “proteins have intrinsic signals that govern their transport and localisation in the cell.” In bacteria there are many proteins that are secreted into the periplasm (*e.g.* OmpA, OmpT, PelB, β -lactamase, alkaline phosphatase) and a key feature of these proteins that determines their fate is that they have a leader or signal peptide sequence. These signal sequences are usually between 15-30 amino acids long and consist of a hydrophobic core with positive residues at the *N*-terminus and polar uncharged residues at the *C*-terminus (von Heijne 1983; von Heijne 1986). The mechanisms by which these signal peptides direct proteins across the bacterial plasma membrane have been extensively studied and reviewed (Baneyx 1999; Driessen, Manting et al. 2001; Rapoport 2007) and can be briefly summarised as follows.

Pre-proteins are synthesised in the cytosol with the signal peptide at the *N*-terminus and are then guided to the translocation machinery at the cytoplasmic membrane by chaperone molecules, either the signal recognition peptide (SRP) or the protein SecB. SRP recognises hydrophobic signal sequences or segments and targets the emerging nascent protein co-translationally. However, most pre-proteins are targeted post-translationally by SecB (§ Figure 3.1).

SecB does not interact with the signal peptide itself but binds to the mature domain of nascent proteins. SecB stabilises the nascent protein in a denatured conformation presumably by allowing the protein to wrap around it. The signal peptide is believed to both prevent folding of the protein, as folded proteins are not translocated, and also play a role in binding the resulting SecB-protein complex to the translocase machinery.

SecB then delivers the protein to the translocase machinery at the membrane of the cell. This consists of a protein pore, SecYEG, and the ATP driven, molecular motor protein, SecA. The SecB-protein complex binds to SecA and repeated cycles of ATP binding and hydrolysis, and protein binding and release, lead to a stepwise translocation of the protein across the membrane. Once the protein has been exported into the periplasm, the signal peptide is then cleaved by a peptidase.

The use of signal peptides has been exploited in recombinant technology in order to direct proteins of interest to the periplasm for various purposes (Baneyx 1999; Sorensen and Mortensen 2005). The use of signal peptide-AT fusions in order to allow secretion of AT in the periplasm of *E. coli*, would potentially enable the development of a life/death selection method for the identification of improved hydrolysis activity AT mutants.

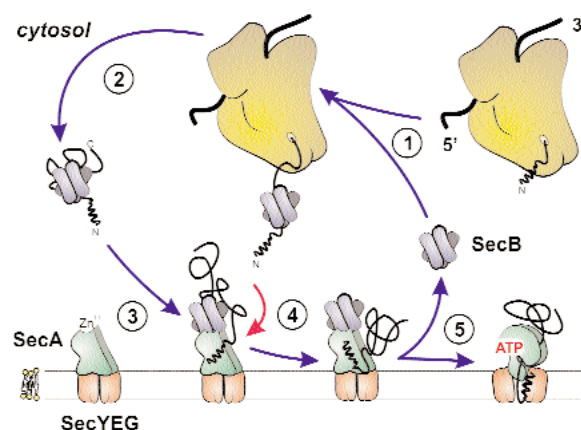


Figure 3.1 Model for Sec-B mediated protein targeting 1.) SecB binds to the mature domain of a nascent protein. 2.) SecB stabilises the protein in denatured conformations. 3.) The SecB-protein complex is targeted to the SecYEG bound SecA. 4.) The signal peptide binds to SecA inducing release of the protein from SecB to SecA. This release is coupled with ATP binding (Driessen, Manting et al. 2001).

3.1.3 Previous Work

Targeting AT to the periplasm using signal peptides had been considered previously in the group (Hart 1996). Firstly, the use of the β -lactamase signal peptide was investigated. The success of this approach had already been demonstrated in the case of chicken muscle triosephosphate isomerase, a cytoplasmic protein. By fusing with the signal peptide of β -lactamase plus the first 12 residues of the mature β -lactamase, chicken muscle triosephosphate isomerase was successfully secreted into the periplasm of *E. coli* (Summers and Knowles 1989). However, when the same approach was taken with AT, only unprocessed, aggregated protein was obtained which did not show any AAT activity (Hart 1996).

The use of maltose binding protein (MBP) was also explored. Like β -lactamase, MBP is a periplasmic protein. Various studies had shown that fusion of various proteins with MBP resulted in the secretion of the protein of interest in the periplasm (Bedouelle and Duplay 1988; Di Guan, Li et al. 1988; Blondel and Bedouelle 1990; Blondel and Bedouelle 1991). The resulting MBP-protein fusions could also be purified using amylose affinity chromatography. MBP-AT fusions were constructed unfortunately, only unprocessed protein which did not show any AAT activity was produced (Hart 1996).

Fortunately, for the author, new crystallographic information became available which allowed us to reconsider the secretion of AT into the periplasm using signal peptides. A salt bridge between the *N*-terminus of AT and the side chain carboxylate of Glu338 was believed to be present, in which case fusion of signal peptides to the *N*-terminus would have implications for the processing and activity of AT (§ Figure 3.2) (Dijkstra 2007).

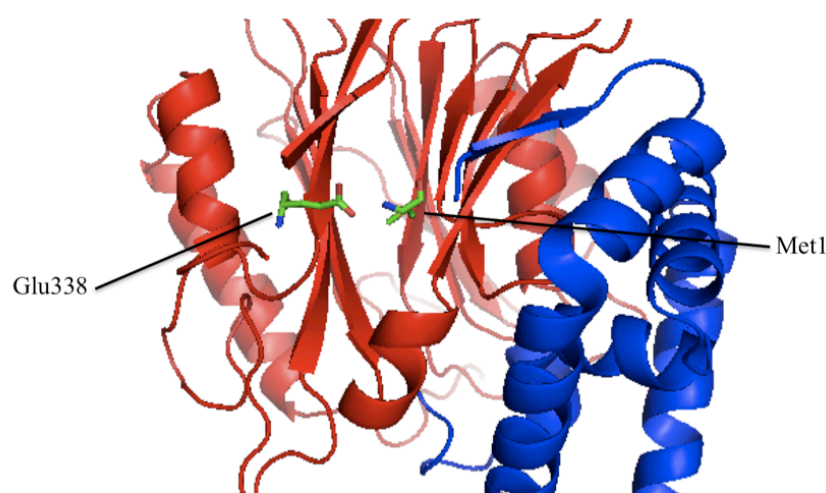


Figure 3.2 To show the location of the salt bridge in AT

3.1.4 The Role of Salt Bridges in Proteins

Salt bridges play an important role in the structure and function of proteins. Surface salt bridges help aid solubility. It has also been implied that salt bridges increase the specificity of folding because there are only a few ways of packing charged groups in a protein while there are many ways of energetically equivalent packing of hydrophobic groups. The presence of charged groups in a protein are also believed to provide suitable environments for catalysis and protein-ligand binding (Bosshard, Marti et al. 2004).

Whether the formation of a salt bridge in a protein is stabilizing or destabilizing is subject to much experimental and theoretical investigation. The observation that thermophilic proteins contain more salt bridges than their mesophilic counterparts would suggest they play a stabilising role, however, there is evidence in favour of both (Waldburger, Schildbach et al. 1995; Kumar and Nussinov 2002).

A salt bridge can be defined as a pH-dependent non-covalent bond between oppositely charged residues that are sufficiently close to each other to experience electrostatic

attraction (Bosshard, Marti et al. 2004). At physiological pH, electrostatic interactions can arise between a pair of ionisable side chains or between ionisable side chains and the termini of the backbone (negative charges from aspartate, glutamate, tyrosine, cysteine or the *C*-terminal carboxylate group, and positive charges from histidine, lysine, arginine or the *N*-terminal amino group).

There are a number of factors that contribute to the strength and stability of a salt bridge in a protein (Pace, Grimsley et al. 2009). The strength of the Coulombic force between the charges depends on pH, the distance between the charges and their geometry. Interactions (*i.e.* van der Waals forces, hydrogen bonds) are also then possible with other ionizable side chains and the dipoles in the peptide bonds of the backbone of the protein and other polar non-ionizable side chains in the vicinity. The stability of a salt bridge is a balance between these interactions and enthalpy effects due to desolvation (the ions must be desolvated before they can interact) and backbone entropy. The location of the salt bridge is also important. Salt bridges on the surface of a protein are subject to an aqueous environment where interaction with and solvation by water are factors, whereas salt bridges buried in the interior of the protein are in a low dielectric, hydrophobic environment (Tanford 1978).

In order to determine the effect of a salt bridge on protein stability, two experimental approaches are used which allow the stability of a protein to be determined by comparing the difference between the free energy of its folded and unfolded state.

The salt bridge can be broken by altering the pH in such a way that the acidic group is protonated or the basic group is deprotonated. This is a non-invasive technique, which gives a relative value. pK_a 's can then be measured using NMR. In one study using this technique, the presence of a salt bridge in T4 lysozyme was found to stabilize the native state by 3-5 kcal mol⁻¹ (Anderson, Becktel et al. 1990).

Site directed mutagenesis can also be used to investigate the effect of salt bridges. Charged residues are mutated to non-ionizable groups and their effect on protein stability determined. However, this technique is not ideal as although the charges are removed the structure of the residue is also altered which can lead to the disruption of other interactions that the side chain played a role in thus altering the protein conformation. This problem can be partially addressed using double mutant cycles (Horovitz and Fersht 1990) where each residue of the pair is mutated individually then

together. This approach is sensitive to the fact that proteins are highly co-operative structures and the interaction between the residues may not be direct but propagated through the molecule; the residues are considered to be coupled to one another. There are number of examples where this approach has been employed to investigate surface salt bridges (Serrano, Horovitz et al. 1990; Strop and Mayo 2000; Marti and Bosshard 2003).

Computational methods have also been successfully employed in analyzing the stabilizing effects of salt bridges. In one example, where the energetics of solvent-exposed ion pairs were analysed, the method employed was found to be accurate to within a few kJ mol^{-1} (Luo, David et al. 1999).

A greater understanding of how salt bridges affect proteins would allow the design of more stable proteins and the subject has been extensively reviewed (Kumar and Nussinov 2002; Bosshard, Marti et al. 2004; Baldwin 2007).

3.1.5 The Salt Bridge in AT and its Implications for MBP-AT Fusions

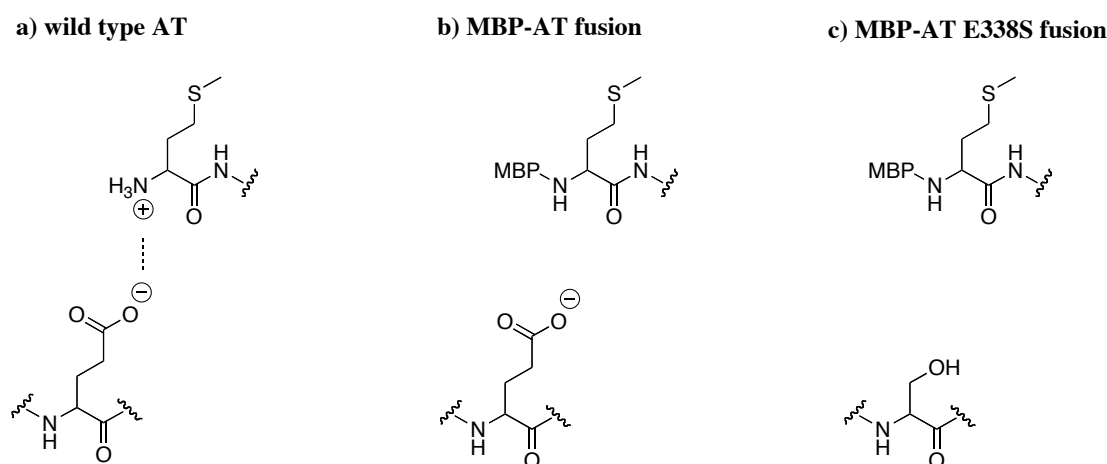
It is generally accepted that the removal of one of the charges from the ion pair that make up a salt bridge decreases the stability of the protein, especially in the case of buried salt bridges as the remaining charge cannot be stabilised due to the hydrophobic environment of the protein core. However, in the case of AT, the salt bridge was observed on the surface of the protein. The removal of a charge from a surface salt bridge is also believed to be destabilising. In one study investigating the electrostatic interactions between the proteins in leucine zippers, it was found that the replacement of a single ion pair partner by an uncharged residue resulted in a less stable dimer (Marti and Bosshard 2003).

The salt bridge in AT is between the main chain nitrogen of Met1 and the side chain carboxylate of Glu338. In a study investigating surface salt bridges in a hyperthermophilic rubredoxin variant, it was found that a main chain to side chain salt bridge was more stabilising than a side chain to side chain salt bridge. It was rationalised that the entropy cost of making a salt bridge with the backbone is less, due to the backbone having been immobilised upon protein folding (Strop and Mayo 2000).

By fusing signal peptides onto the *N*-terminus of AT it is apparent that the salt bridge had been disrupted, hence affecting the stability of the resulting signal peptide-AT fusion. This would explain the previous observations of unprocessed, inactive AT for both the β -lactamase and MBP protein fusions (Hart 1996).

The presence of the signal peptide on the *N*-terminus would remove the positive charge of the ion-pair making up the salt bridge, leaving the negative charge exposed. One way to remove this negative charge would be to replace the Glu338 residue for a neutral residue using site directed mutagenesis in order to hopefully restore the stability to AT.

As has already been discussed, when removing a charge by substitution with another residue there are other subtleties to be aware of, interactions the side chain was involved with and the effect on the overall conformation of the protein. To replace Glu338, a non-ionisable, neutral residue was required with some consideration to sterics. A range of substitutions could have been made and their effect studied in order to find the ideal candidate. However, when discussing the problem, the crystallographer was confident that substituting glutamate for serine was the obvious choice (Dijkstra 2007). The salt bridge between the *N*-terminus (Met1) and Glu338 and the effects of MBP fusion and Glu338Ser mutation are depicted below in Scheme 3.1.



Scheme 3.1 To show the effect of MBP and Glu338Ser on the salt bridge of AT

Either of the previous signal peptide-AT fusion strategies for secreting AT into the periplasm of *E. coli* could have been revisited. However, it was decided to reinvestigate the MBP-AT fusions and not the β -lactamase-AT fusions.

There are advantages to using MBP over β -lactamase. Firstly, the resulting MBP-AT fusion can be purified using amylose affinity chromatography. Also, it has been shown that the presence of MBP enhances the solubility of proteins minimising inclusion body formation (Kapust and Waugh 1999). Another advantage is that MBP does not contain any cysteine residues that could interfere with disulfide bond formation within the target peptide (Di Guan, Li et al. 1988).

3.1.6 Construction of *malE-penDE* fusion plasmid pHAR-4p

The plasmid (pHAR4-p) used in this work was constructed by previous group member Darren Hart (Hart 1996) as follows. The commercially available pMAL-p2 vector from New England Biolabs® Inc was used. This contains the *malE* gene, which encodes MBP and is under the control of a strong tac promoter. A multiple cloning site (MCS) and polyasparagine linker are also present, fused onto the 3' end of the *malE* gene allowing the gene of interest to be fused directly. There is also a recognition site for the protease Factor Xa, which allows the protein of interest to be cleaved from the MBP after purification if required.

The *penDE* gene was excised from another plasmid by first of all changing the *Nde* I restriction site at the beginning of the gene to an *Sph* I site using site directed mutagenesis, then digesting with *Sph* I. The resultant overhangs were blunt ended with T4 DNA polymerase. Unfortunately, this led to the removal of Met1 from AT. The *penDE* blunt end/*Hind* III fragment was then ligated into the pMAL-p2 backbone, which had been digested using *Xmn* I (blunt ends) and *Hind* III.

The pMAL-p2 vector also contains a gene (*bla*) encoding for ampicillin resistance. The presence of β -lactamase was undesirable as it can also hydrolyse β -lactam antibiotics, therefore the gene was inactivated by inserting a commercially available chloramphenicol resistance cassette. *E. coli* contains chromosomally encoded β -lactamase which one had to be aware of when using crude protein extracts. However, when purified using amylose affinity chromatography this would not be a problem, as the β -lactamase would not be retained by the amylose resin.

There were implications to the inadvertent removal of Met1. By placing the MBP onto the N-terminus of AT it was believed that the salt bridge in which it had participated had been severely disrupted leading to unprocessed inactive AT. However, perhaps it

was a combination of both the MBP fusion and the removal of Met1 that was so detrimental to the processing and activity of AT and maybe if the MBP had been fused directly onto a methionine at position 1, as had been supposed, the effect would not have been so catastrophic. Therefore it was decided that as well as mutating Glu338 to serine, Met1 would also be restored using site directed mutagenesis.

As a result of the cloning process, position 1 in AT was now effectively the final amino acid in the polylinker, an arginine residue. This was also part of the Factor Xa recognition site. Factor Xa cleaves the amino acid sequence Ile-Glu/Asp-Gly-Arg directly after the arginine residue, so making this mutation would forego the possibility of cleaving the MBP from AT if required. However, it had been found previously that the presence of MBP on the C-terminus of AT (plasmid pHAR10a) did not hinder activity so cleavage was not necessary (Hart 1996). It was anticipated that this would also be the case when MBP was fused onto the N-terminus of AT.

Coincidentally, it should be noted that as an ionisable side chain, arginine exists as a positive ion at physiological pH. Arginine is the most basic amino acid. This basicity is due to the presence of the guanidinium group at the terminus of arginine's side chain. It has been found that the stability of salt bridges increase as the basicity of the amino acid also increases (Prell, O'Brien et al. 2009). However, in the case of AT the positive ion required for the salt bridge formation comes from the backbone nitrogen of the amino acid at position 1 and not the side chain. The possibility of the positive charge of the Arg1 side chain restoring the positive ion pairing of the salt bridge is remote due to the rearrangement in the conformation of the amino acid that would be required in order for it to act as a counter ion to the negative charge of Glu338. One assumes that as unprocessed, inactive AT was obtained that this indeed did not occur.

3.2.1 Verification of plasmid pHAR4-p

As the plasmid pHAR4-p had been in storage for a number of years, preliminary experiments sought to verify its integrity. The plasmid had been stored as a precipitate and was therefore re-suspended in sterile Milli-Q H₂O and the concentration of the resulting solution determined by agarose gel electrophoresis (§ 6.2.3). The plasmid was then transformed into XL1-Blue supercompetent cells using the heat shock method (§ 6.3.4) and glycerol stocks prepared.

Growth and harvestation of the cells (§ 6.4.1) enabled DNA to be isolated which was then analysed by restriction digest. The plasmid cut as expected and the results were also compared with the previous work, which was reproduced.

3.2.2 Site Directed Mutagenesis of pHAR4-p

Site directed mutagenesis (§ 6.3.1) was performed on pHAR4-p to obtain Arg1Met and Glu338Ser. The mutations were verified by restriction digest *vide supra* and DNA sequencing (§ 6.2.5. For primers see Appendix D).

3.2.3 MBP-AT Fusion Protein Expression and Analysis

Proteins were expressed from pHAR4-p (wild type), Arg1Met and Glu338Ser. Due to the smaller volume of the periplasm compared to the cytoplasm, protein expression was carried out on a larger scale, 5× 100 mL as opposed to the 1× 100 mL scale carried out for cytoplasmic expression of AT (§ Chapter 2). The resulting cell pellets from subsequent growth and harvestation were fractionated in order to obtain the cytoplasmic and periplasmic fractions separately (§ 6.4.5).

SDS-PAGE (§ 6.4.8) analysis of the crude single mutants Arg1Met and Glu338Ser cytoplasmic and periplasmic proteins showed lots of protein bands making it difficult to identify the bands corresponding to the MBP-AT fusion protein *i.e.* 51 kDa for the MBP- α subunit (40 + 11 kDa) and 29 kDa for the β -subunit.

The activity of the crude AT mutant proteins with respect to the formation of Pen G [5] from 6-APA [1] and PANAC [37] was determined (§ 6.6.3). The formation of Pen G [5] from 6-APA [1] was selected for this purpose as it is the natural reaction of wild type AT. Both single mutants, Arg1Met and Glu338Ser, showed activity. It was found for both Arg1Met and Glu338Ser that the crude cytoplasmic protein gave greater kill zones than the crude periplasmic protein. The mutant Glu338Ser also gave slightly larger kill zones than the Arg1Met mutant for both the cytoplasmic and periplasmic fractions. However, as crude protein preparations were used, these differences in activity may have been due to differences in the actual amount of AT present, which had not been quantified.

These initial results were very encouraging. Although activity was observed for both the cytoplasmic and periplasmic fractions of the single mutants, it was not observed for

either of the protein fractions obtained from pHAR4-p (wild type). This suggested that activity had been restored to AT by the mutations made. However, it was not clear at this stage whether AT had been successfully targeted to the periplasm although previous work would suggest it had (Hart 1996). The results also show that the presence of MBP at the *N*-terminus of AT has no effect on its activity as had been anticipated due to the observation that when MBP was present at the *C*-terminus of AT it was still active (§ Chapter 2).

As active AT was formed it would also suggest that the salt bridge was not essential for correct folding of AT. It is also speculated that any solubility properties that the surface salt bridge may have imparted to AT were perhaps compensated for by the presence of MBP, which is known to enhance the solubility of proteins *vide supra*.

The observed activity for the Arg1Met mutant was quite surprising. The side chain of any residue at position 1 was not deemed to have any role in the salt bridge; it was the backbone of the amino acid at this position that played a role in the salt bridge. By placing the MBP here it had prevented it from carrying out its role, leaving a destabilising lone negative charge at Glu338. However, arginine is also charged and perhaps the positive charge of the arginine side chain also had a destabilising effect on AT the effect of which was neutralised by this mutation.

In the case of the Glu338Ser mutant, replacing the negative glutamate residue with the neutral serine residue had the desired effect; the instability due to the presence of the lone negative charge at this position had indeed been mitigated.

The crude protein preparations were also purified using affinity chromatography (§ 6.4.6). However, presumably due to the scale of protein employed, loading and elution of protein to and from the column was difficult. Initial SDS-PAGE analysis showed very faint protein bands, which were difficult to assign with certainty. The extended length of the protein purification procedure due to handling issues also lead to doubts to the integrity and stability of any resulting protein.

As the removal of one destabilising charge, either positive as in the case of Arg1Met or negative as for Glu338Ser, restored activity to AT, it was anticipated that the removal of both the charges *via* the double mutant Arg1Met Glu338Ser, would lead to AT with greater activity than that of either mutation alone. Site directed mutagenesis was carried

out to obtain the double mutant Arg1Met Glu338Ser. The single mutant Arg1Met was used as the parent and the primers for the Glu338Ser were again employed.

Proteins were expressed for the following variants, pHAR10a (control), pHAR4-p (wild type), Arg1Met, Glu338Ser, and Arg1Met Glu338Ser on a 5× 100 mL scale and the resulting cell pellets were fractionated in order to obtain the periplasmic fractions as before (§ 6.4.5). The resulting periplasmic fractions were then concentrated using Amicon Ultra-15 centrifugal filter units (Millipore™). Although the use of the filters is recommended for the concentration of dilute or pre-purified proteins column eluents, in this case they were used before column purification in order to achieve quicker loading times. However, in practise this concentration step did not significantly improve column loading times. The periplasmic proteins were purified by amylose affinity chromatography as before then analysed by SDS-PAGE. Faint bands were observed at the expected 51 kDa (40 kDa MBP + 11 kDa α -subunit) and 29 kDa (β -subunit) (gel not shown). Bradford assay to determine the concentrations of the various proteins revealed relatively low values, (ranging from 0.144 – 0.244 $\mu\text{g } \mu\text{L}^{-1}$) especially for the double mutant Arg1Met Glu338Ser (0.058 $\mu\text{g } \mu\text{L}^{-1}$). Expression of cytoplasmic AT-MBP from 100 mL of cell culture typically yielded approximately 1.5 $\mu\text{g } \mu\text{L}^{-1}$ (§ Chapter 2).

The activity of the concentrated and purified AT mutant proteins with respect to the formation of Pen G [5] from 6-APA [1] and PANAC [37] was determined as before. However, none of the mutant proteins showed any activity. Proteins with equally low concentrations had shown activity in other areas of the work (§ Chapter 2). The lack of activity was presumed to be due to the combination of the long protein purification times and the use of the concentrating filters the effects of which were detrimental to the protein's activity. Although neither of these would be factors in any assay, the low yields of protein obtained from the periplasm compared to cytoplasm however did give cause for concern. For this reason as well as the high cost of IPN [18], a library of MBP-AT variants was not created nor subsequently screened.

3.3 Chapter 3 Summary

The use of the signal peptide, MBP, to target AT to the periplasm of *E. coli* was reinvestigated in order to allow the development of a life or death selection method for the identification of improved hydrolysis activity AT mutants.

A salt bridge between Met1 and Glu338 had been observed which had implications for the fusion of any signal peptide to the *N*-terminus of AT. Site directed mutagenesis was used to introduce mutations to AT that would compensate for the presence of the signal peptide. The single mutant Arg1Met was made in order to restore the methionine removed during the cloning process. Glu338Ser was made to neutralise the lone negative charge that arose due to the presence of the signal peptide. The double mutant Arg1Met Glu338Ser was also made and investigated.

The mutations did restore activity to AT. However, the concentration of protein was thought to be too low to allow MBP-AT fusions to be employed for the identification of improved hydrolysis activity AT mutants.

CHAPTER FOUR

Investigations into β -Lactamase

Towards an Approach for Hydrolysis

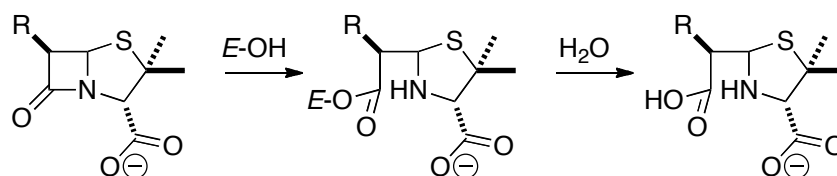
Screening of AT

4.1 Introduction

As the periplasmic expression of AT had been deemed unsuitable for use as part of a selection method for identifying AT mutants with increased activity for the hydrolysis of IPN [18], another approach was sought.

As already discussed (§ Chapter 3), a major problem when considering the hydrolysis step independently from the acylation step is that once IPN [18] is hydrolysed, 6-APA [1] remains in the active site of AT awaiting re-acylation; 6-APA [1] effectively acts as an inhibitor of AT. One way to overcome this problem would be to somehow remove 6-APA [1] from the active site of AT thus allowing AT to turn over more molecules of IPN [18], the higher concentration of the resulting hydrolysed product would be easier to detect in any screening or selection method. To this end the use of a β -lactamase was investigated.

β -Lactamases are a group of bacterial enzymes that cleave and hydrolyse the β -lactam ring of β -lactam antibiotics (§ Scheme 4.1). They target the bacterial cell wall and are responsible for the resistance of bacteria to this class of antibiotic. Their role in the resistance of bacteria to β -lactam antibiotics has already been discussed (§ 1.4.1).



Scheme 4.1 Cleavage of a β -lactam by a β -lactamase

It was hoped that the presence of a β -lactamase during a screen of AT variants would hydrolyse 6-APA thus freeing up the active site of AT. However, the β -lactamase used would have to be selective for 6-APA [1] only and not for IPN [18]. If β -lactamase also hydrolysed IPN [18] this would lead to the identification of false hits from the screen. The survival of colonies screened in the presence of IPN [18] would therefore be due to the hydrolysis of IPN [18] by AT and not β -lactamase and any colonies that did survive the screen would hopefully contain AT variants with improved capability for the hydrolysis of IPN [18].

4.1.1 RTTEM-1 β -Lactamase

There are four different classes (A-D) of β -lactamases based on primary sequence homology. Class B β -lactamases are metallo-enzymes that use zinc ions to hydrolyse the β -lactam ring, whereas classes A, C and D β -lactamases utilize a catalytic serine as the primary nucleophile. RTTEM-1 β -lactamase is a class A enzyme and is the most common plasmid-encoded β -lactamase in Gram-negative bacteria (Brown, Shanker et al. 2009).

A more recent classification system where β -lactamases are grouped according to their hydrolytic and inhibition properties is also in use in clinical settings. According to this classification system RTTEM-1 falls into subgroup 2b. Subgroup 2b β -lactamases readily hydrolyze penicillins and early cephalosporins, such as cephaloridine [34] and cephalothin [15], and are strongly inhibited by clavulinic acid [17] and tazobactam [35] (Bush and Jacoby 2010). Unfortunately for the author there is no comparison of the susceptibility of the various β -lactamases to 6-APA [1].

The nucleotide sequence of the ampicillin resistance gene of *Escherichia coli* plasmid pBR322 (penicillin amide- β -lactamhydrolase EC 3.5.2.6) has been determined (Sutcliffe 1978) and in this work the standard numbering scheme for the class A β -lactamases was employed when referring to the residues of RTTEM-1 β -lactamase (Ambler, Coulson et al. 1991). In the case of RTTEM-1, the leader sequence is 23 amino acids long and numbering starts at three, therefore the first amino acid in the mature protein is His26.

4.1.2 The Natural Selectivity of β -Lactamase for 6-APA

Initial work on this approach began by looking at the natural selectivity of β -lactamase for 6-APA [1]. The commercially available Penicillinase (EC 3.5.2.6) from *Bacillus cereus* β -lactamase (Sigma®) was used. Solutions (12.3 mM, 1.5 mL) of both 6-APA [1] and Penicillin G [5] were incubated with the β -lactamase (2 mg) at 25°C overnight then analysed by ^1H NMR. Control experiments without any β -lactamase were also carried out. Hydrolysis of Penicillin G [5] by β -lactamase was evident as was the hydrolysis of 6-APA [1] albeit it lower than that of Penicillin G [5]. Although the

results showed that β -lactamase did have some activity for 6-APA [1] the effect on *E. coli* could not be deduced.

The resistance of *E. coli* to 6-APA [1] inferred by the presence of β -lactamase was determined by plating XL1-Blue cells containing the pHAR10a plasmid, *i.e.* no β -lactamase present, and cells containing the pUC18 plasmid onto various concentrations of 6-APA [1]. pUC18 is a commercially available plasmid which contains the *amp*^R gene encoding β -lactamase (Sutcliffe 1978). The MIC of 6-APA [1] for cells with and without β -lactamase were 10 mM and 3.5 mM respectively. This showed that the presence of the β -lactamase increased the MIC of the *E. coli* cells to 6-APA [1] by 6.5 mM.

In order to increase the sensitivity of the assay for hydrolysis mutants in the presence of β -lactamase the use of the *E. coli* ESS strain was investigated. *E. coli* ESS had been used as an indicator strain throughout the project as it had previously been found to be a thousand times more sensitive to Penicillin G [5] than XL1-Blue (Derome 2003). It was anticipated that if this *E. coli* strain could be used in tandem with β -lactamase a highly sensitive hydrolysis assay would result.

In order to clarify the suitability of *E. coli* ESS as a host organism, competent *E. coli* ESS cells were prepared (§ 6.3.6). Transformation of both pHAR10a and pUC18 into *E. coli* ESS was then attempted. Transformation of XL1-Blue cells with both pHAR10a and pUC18 was also carried out concurrently as a control experiment. Unfortunately, transfection into *E. coli* ESS could not be achieved using either heat shock or electroporation methods (§ 6.3.4 and § 6.3.5).

It was not clear why it was not possible to transfect *E. coli* ESS. It was thought that it might be due to differences in the cell wall structure of *E. coli* ESS compared to that of XL1-Blue. During transfection the cell wall is temporarily disrupted to allow the negatively charged DNA molecules to pass through the cell membrane. In the case of cells with a weaker cell wall, as is supposed in the case of *E. coli* ESS, this process may be detrimental to the cell. These cell wall differences would also account for the increased susceptibility of *E. coli* ESS to β -lactam antibiotics, which are known to inhibit the growth of bacteria by inhibiting cell wall formation (§ 1.3).

As *E. coli* ESS could not be transfected with either pHAR10a or pUC18, other ways to increase the sensitivity of the assay were considered. As had already been shown by ¹H NMR and MIC determination experiments, β -lactamase could inactivate 6-APA [1] by hydrolysis. However, it was wondered if this could be improved further using directed evolution.

4.2 Directed Evolution of RTEM-1 β -lactamase

In a well-known example of directed evolution, β -lactamase was used as a model system for mutagenic DNA shuffling for molecular evolution. Repeated cycles of shuffling and backcrossing were each followed by selection on increasing concentrations of the antibiotic cefotaxime [36] resulting in mutants with an increased MIC of 640 $\mu\text{g mL}^{-1}$, a 32,000-fold increase on wild type (Stemmer 1994). This work shows that the substrate specificity of β -lactamase can be altered using directed evolution.

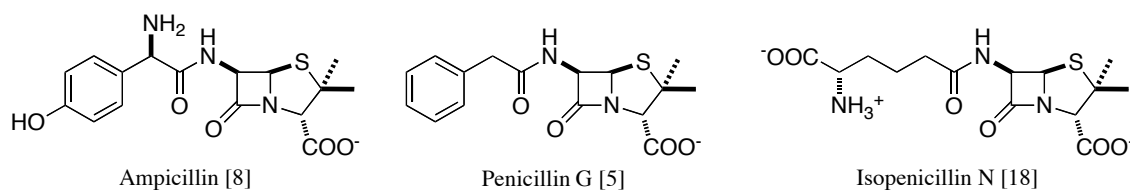
A β -lactamase that had high selectivity for 6-APA [1] and no selectivity for IPN [18] was desired. By having a high selectivity for 6-APA [1] the sensitivity of the screen would be increased as the amount of 6-APA [1] removed from the active site of AT would be greater thus allowing AT to turnover more molecules of IPN [18]. If the same β -lactamase variant could not hydrolyse IPN [18] any hydrolysis would be due to AT present only and not the β -lactamase. However, it was expected that some hydrolysis of IPN [18] by β -lactamase would be inevitable and a mutant with a high specificity for 6-APA [1] and low specificity for IPN [18] was the goal.

4.2.1 Random Approach

4.2.1.1 Strategy

A random library of β -lactamase variants was generated and screened. Due to the high cost of IPN [18] initial screening was carried out in the presence of 6-APA [1] and the antibiotics ampicillin [8] and Penicillin G [5]. According to the First law of Directed Evolution ('You get what you screen for') (Arnold 1998) therefore this was a slightly risky strategy especially when the side chains of ampicillin [8], Penicillin G [5] and IPN [18] are compared (§ Scheme 4.2). Ampicillin [8] and Penicillin G [5] have aromatic side chains whereas IPN [18] does not. However, in terms of initial screening this was

considered sufficient, as a mutant with no binding capability for any side chain as in the case of 6-APA [1] was desired.



Scheme 4.2 Side chains of ampicillin [8], Penicillin G [5] and IPN [18]

4.2.1.2 Library Generation

To create a library of β -lactamase mutants the commercially available mutator strain XL1-Red (Stratagene) was used (§ 6.3.2). This method involves propagating the cloned gene into XL1-Red, an *E. coli* strain which is deficient in three of the primary DNA repair pathways. The random mutation rate in this triple mutant is approximately 5000-fold higher than that of wild type. The main advantage of this method is that it does not require extensive genetic or biochemical manipulation and as such is a rapid method of library generation.

The supplied protocol was carried out as follows. pUC18 was first transformed into the XL1-Red cells using the heat shock method (§ 6.3.4). After a short incubation period (37°C, 1 hour) the cells were harvested and plated on LB agar supplemented with ampicillin (50 mg L⁻¹). After overnight incubation the resulting colonies were washed off the plates with LB media to produce a stock cell culture that was used to inoculate LB media containing various concentrations of 6-APA [1] (0, 6, 8, 10 and 12 mM). After a period of incubation (18 hours, 37°C, 250rpm), the OD₆₀₀ of the cell cultures was measured and the results were as shown in Table 4.1.

6-APA /mM	OD ₆₀₀
0	1.1340
6	0.9287
8	0.4973
10	0.2757
12	0.0172

Table 4.1 To show the OD₆₀₀ values obtained for transformants grown at various 6-APA [1] concentrations.

As can be seen from the data in the table the cell density of the cultures decreased as the concentration of 6-APA [1] increased. It was anticipated that by growing the transformants in the presence of 6-APA [1] and not ampicillin [8] some selection would be observed, which would give an indication of the hydrolysis capability of the mutants present in the library.

The DNA was isolated from the harvested cells (§ 6.2.1). The DNA obtained from the cells grown at 8, 10 and 12 mM 6-APA [1] was combined. The plasmid DNA was analysed by agarose gel electrophoresis after single site digest by the restriction endonuclease NdeI (§ 6.2.4). The size of the resulting band (2,686 bp expected) was confirmed by comparison to a DNA marker (*not shown*).

4.2.1.3 Screening of the Library

The DNA libraries were transformed into XL1-Blue (§ 6.3.4) and the resulting cells plated onto 6-APA [1] (12 and 15 mM). Cells were also plated onto ampicillin [8] (50 mg L⁻¹) LB agar plates. After a period of incubation (18 hours, 37°C) colonies were seen to have only formed on the ampicillin [8] plates. Ten of these clones were selected and the region containing the *amp*^R gene was sequenced (§ 6.2.5. For primers see Appendix D), however no mutations were found. Mutator strains introduce random mutations into the entire plasmid; the mutations introduced are not limited to the gene of interest. As no mutations were found in the *amp*^R gene, which would explain the survival of the colonies, it was assumed that mutations in the promotor region were

responsible for the survival of these colonies perhaps by increasing the amount of β -lactamase present in the colonies.

The non-appearance of mutants on either 12 or 15 mM 6-APA [1] was believed to be due to increased sensitivity of the transformants to 6-APA [1]. It had previously been found that the MIC of XL1-Blue with pUC18 was 12 mM 6-APA [1] therefore the library would be plated onto lower concentrations of 6-APA [1].

The library was transformed into XL1-Blue (100 μ L) as before and the resulting cells plated onto 10 mM 6-APA [1] agar plates (4 \times 22.5 \times 22.5 cm). This resulted in many colonies, which were too numerous to count. The colonies were washed off the plates using liquid media to give a stock cell culture, which was diluted 100-fold then plated onto various concentrations of 6-APA [1] (11, 11.5, 12 and 12.5 mM) agar plates (6 \times 22.5 \times 22.5 cm). On the 11, 11.5 and 12 mM plates numerous colonies appeared. At 12.5 mM 6-APA [1] 200 colonies were obtained from the approximately \sim 21,000 colonies plated at 12.5 mM.

Of these 60 were picked at random and tooth picked onto agar plates containing either ampicillin [8] (50 mg L⁻¹) or 6-APA [1] (12.5, 13, 15 or 20 mM). Of these 11 colonies were killed at 20 mM. All 60 survived in the presence of ampicillin [8]. These 11 colonies were sequenced to determine if there was any difference in the *amp*^R gene that lead to this result. However, no mutations were found in the *amp*^R gene.

The 60 colonies were again tooth picked onto agar plates containing either ampicillin [8] (100 mg L⁻¹) or 6-APA [1] (25 mM). Of these 4 did not survive the higher concentration of 6-APA [1] but all survived the higher ampicillin [8] concentrations. These 4 were sequenced however the data did not reveal any differences in the *amp*^R gene.

Although the colonies selected so far contained had improved resistance to both 6-APA [1] and ampicillin [8], sequencing again revealed no mutations present in the *amp*^R gene which would explain the increased resistance. Clearly the random nature of the library generation did not give the desired effect therefore another approach was needed.

4.2.2 Rational approach

As the random approach to mutagenesis of β -lactamase had been unsuccessful a rational approach was investigated. As the goal was to find a mutant with a high specificity for 6-APA [1] and a low specificity for IPN [18] it was reasoned that the substrate binding capabilities of β -lactamase should be targeted and the residues responsible for binding the side chain of the β -lactam antibiotics would be of interest.

In order to identify the residues responsible for binding the side chains of β -lactams a crystal structure of β -lactamase was needed with a β -lactam substrate *in situ*. Due to the clinical importance of β -lactamases and their detrimental effects on β -lactam antibiotics much work towards elucidating their structures and determining their catalytic mechanism has been carried out and a number of crystal structures have been reported at various resolutions (Herzberg and Moulton 1987; Knox and Moews 1991; Jelsch, Lenfant et al. 1992). β -lactamase consists of two domains connected by two hinge regions. The first domain consists of a five-stranded β -sheet and the second domain is made up of eight helices.

4.2.2.1 Crystal Structure of RTE-1

The X-ray crystal structure of the deacylation-defective mutant (Glu166Asn) of RTE-1 β -lactamase with and without Penicillin G [5] at 1.7 Å resolution has been reported and compared with that of native RTE-1 (Strynadka, Adachi et al. 1992). The mutant Glu166Asn had been previously found to be deacylation defective (Adachi, Ohta et al. 1991). Accumulation of a covalent complex with Penicillin G [5] occurs and the acyl-enzyme intermediate obtained has the required stability for crystallographic studies. The complex formed consists of Penicillin G [5] trapped by the RTE-1 Glu166Asn mutant *via* covalent bond between C7 of Penicillin G [5] to Ser 70 O^γ as shown in Figure 4.1 below.

The interactions described below are believed to stabilize the initial binding of Penicillin G [5]. There are two hydrogen bonds between the carbonyl oxygen atom O8 to the main chain nitrogen atoms of Ser70 (2.7 Å) and Ala237 (3.0 Å). These interactions make up the oxyanion binding site of the enzyme. The nitrogen N14 and oxygen O16 atoms of the side chain peptide on Penicillin G [5] form hydrogen bonds

with the main chain carbonyl oxygen atom of Ala237 (2.9 Å) and the amide nitrogen of Asn132 N^{δ2} (2.6 Å) respectively.

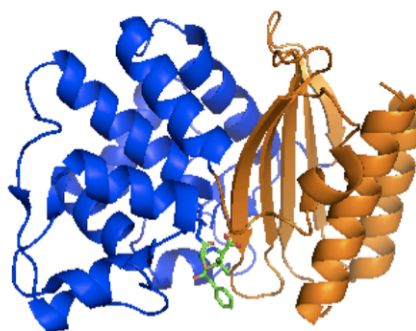
The carboxylate oxygen atoms O12 and O13 of Penicillin G [5] form strong hydrogen bonds to the side chain nitrogen atom of Arg244 N^{η1} and the side chain oxygen atom of Ser235 O^γ. There is a very weak interaction between the carboxylate carbon atom C3 and Lys234 N^ζ (3.5 Å). The distance between the Ser130 O^γ and N4 of thiazolidine ring of Pen G [5] is 3.1 Å. However, the orientation does not allow a hydrogen bond, in which N4 would be the donor atom, only van der Waals interaction.

A crystal structure of an acylation transition state analog has also been reported which goes towards clarifying some of the mechanistic implications (Maveyraud, Pratt et al. 1998). The residues directly involved in catalysis are Ser70, Lys73, Ser130 and Glu166 (carboxylate is the general-base catalyst). The charged groups on Lys234 and Arg244 stabilise intermediates and therefore act as electrostatic catalysts. These residues are also shown in Figure 4.1.

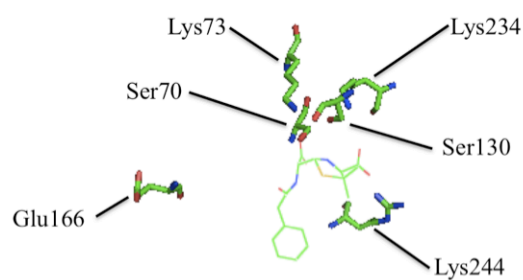
4.2.2.2 Mechanism of catalysis

There are two stages in the catalytic mechanism of class A β -lactamases. Acylation followed by deacylation. In the acylation step a proton is removed from Ser70. The resulting oxygen anion of Ser70 then attacks the carbonyl of the β -lactam resulting in an acyl-intermediate. This acyl-intermediate then collapses with the amide nitrogen picking up a proton from a coordinated water molecule. Attack of the acyl-intermediate by a water molecule activated by Glu166 and protonation of Ser70 completes the hydrolysis of the β -lactam and regenerates the enzyme. This mechanism is based on the acyl-enzyme intermediate shown in Figure 4.1.

a)



b)



c)

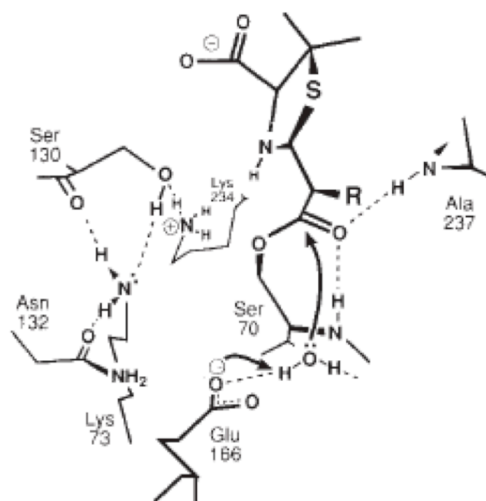


Figure 4.1 a) An overview of β -lactamase (blue) with Penicillin G [5] in the active site (PDB: 1FQG); b) Catalytic residues of β -lactamase (sticks) in relation to Penicillin G [5] (lines) (PDB: 1FQG); c) Acyl-enzyme intermediate (Strynadka, Adachi et al. 1992).

4.2.2.3 Selection of Residues for Mutation

Residues implicated in the mechanism were not considered for mutation. However the stabilizing residues described above were. The crystallographic data was downloaded from the Protein Data Bank (PDB ID: 1FQG) and viewed using MacPyMOL, a molecular viewing system, in order to help select suitable residues for mutation. Any residues that appeared to interact with the side chain of Penicillin G [5] were considered while those that interacted with the penam nucleus were not.

It appeared that Pen G [5] enters β -lactamase *via* the penam nucleus end first so when considering residues to mutate it was wondered whether a molecular sieving approach could be affected. That is to say increasing sterics at pertinent positions would effectively block larger molecules from entering β -lactamase so in terms of selecting 6-APA [1] over Pen G [5], 6-APA [1] would be selected due to its smaller size. However, this may also depend on the substrates used for screening *vide supra*.

After much consideration of the β -lactamase structure and its interactions with Penicillin G [5] in MacPyMOL, two residues were selected for mutation, Asn132 and Ala237.

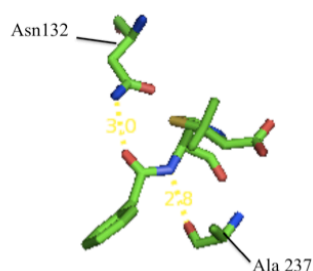


Figure 4.2 The interaction of Asn132 and Ala237 with Penicillin G [5] (PDB: 1FQG).

4.2.2.4 Site Saturation Mutagenesis

Site saturation mutagenesis (§ 6.3.1) was performed on pUC18 to obtain two separate libraries with a range of mutations at Asn132 and Arg237 (for primers see Appendix D).

Transfection of XL1-Blue with the pUC18 libraries and subsequent plating on 8 mM 6-APA [1] gave approximately 100 colonies per library. These colonies were tooth picked onto agar plates containing 30 mM 6-APA [1], 100 mM Pen G [5] or 100 mM ampicillin [8] and incubated (18 hours, 37°C). The colonies showed various characteristics in terms of resistance to the three substrates.

4.2.2.5 Screening the Asn132 and Ala237 Libraries

Potentially there are 8 resistance scenarios depicted in Figure 4.3 below. Colonies that were resistant to the higher 6-APA [1] concentrations and not resistant to Penicillin G [5] or ampicillin [8] were desired. However, as a result of screening colonies with these characteristics did not arise. The colonies certainly had different selectivities compared to one another and some were selected for sequence determination in order to try and ascertain some clues that would account for these differences.

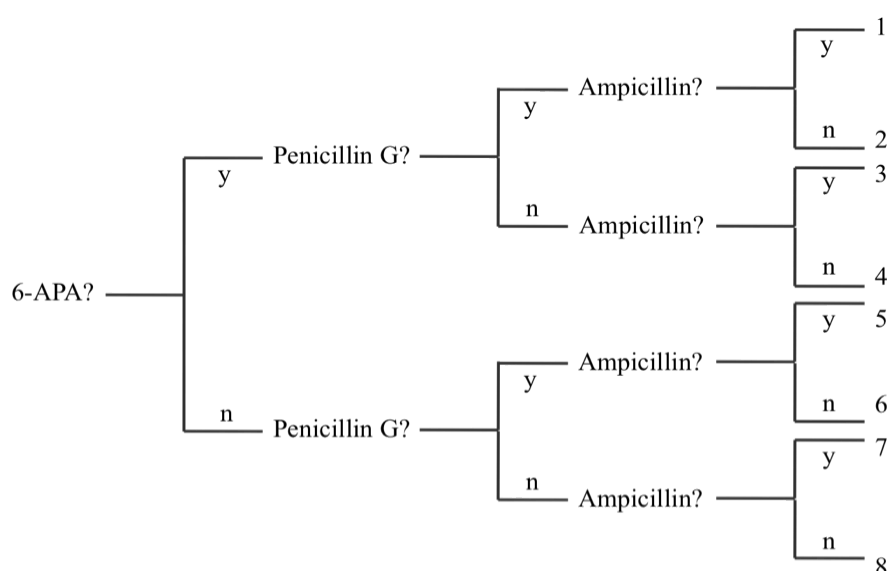


Figure 4.3 Resistance profile of β -lactamase variants

Colonies with resistance profiles 3 and 8 (as shown in Figure 4.3) were selected from both libraries as this gave manageable numbers for sequence determination. The results are shown in Table 4.2.

Resistance Profile	Total No.	Library	No.	Phenotype
3	13	N132X	8	1× N132N
				7× wt
		A237X	5	1× A237A
				4× wt
8	9	N132X	1	1× wt
				-
		A237X	8	7× A237G
				2× wt

Table 4.2 Sequencing results for variants obtained from screening Asn132 and A237 libraries

Of the 21 colonies selected for sequence determination only three contained mutations and two of these were silent. The mutant found was Ala237Gly and was not resistant to the higher concentrations of any of the substrates used under the screening conditions. Tooth picking experiments to compare the mutant with wild type β -lactamase were carried out at various concentrations of 6-APA [1] (10 mM, 20 mM, 30 mM and 40 mM) and ampicillin [8] (50 and 100 mg L⁻¹) not used during the screening process, unfortunately there was no difference in their resistance, which confirmed the screening results.

When the mutant was considered again in the context of its interaction with Penicillin G [5], it was perhaps not surprising that this mutation had no effect on the substrate specificity of the mutant as it is the main chain nitrogen and oxygen atoms of Ala237 that interact with Penicillin G [5] *via* the carbonyl oxygen O8 and the nitrogen N14 respectively; substituting an alanine for a glycine residue was not sufficient to alter the interaction as desired.

Despite repeated attempts at the screening process, no key mutations towards the goal of increased 6-APA [1] activity and reduced acylated penicillins were obtained from either the Asn132 or Ala237 libraries. This was disappointing as the rational approach made possible by the crystallographic data augured well.

When the amino acid sequences for various class A β -lactamases (Ambler, Coulson et al. 1991) were compared it was observed that at position 132 Asn is conserved through all 20 examples. This would suggest that this is a key residue. In the case of position 237, Ala dominated ($\times 11$) however Gly was also present ($\times 4$) as well as Glu ($\times 2$), Thr ($\times 2$) and Ser ($\times 1$), which would perhaps suggest that this position is not as crucial. The mutant Ala237Gly identified from the screening process was therefore homologous.

Further literature searches revealed that Asn132 is in fact a key residue in binding the side chain of β -lactam antibiotics. In one study each residue of RTTEM-1 β -lactamase was mutagenised in order to identify those that are essential to its structure and function. 43 out of the 263 amino acid residues were determined to be essential. Asn132 belonged to a sub group of these essential residues that were believed to make important interactions for substrate binding or active site conformation (Huang, Petrosino et al. 1996).

In another study, conserved β -lactamase active site residues were mutated and screened on ampicillin [8], which led to the identification of Asn132Asp. This mutant was found to have a decreased k_{cat} for Penicillin G [5] and ampicillin [8] but an increased k_{cat} for 6-APA [1] (Osuna, Viadiu et al. 1995).

4.2.2.6 Generation of Asn132Asp

Site directed mutagenesis (§ 6.3.1) was carried out in order to create the β -lactamase mutant Asn132Asp described in the literature *vide supra* (for primers see Appendix D). Subsequent sequencing (§ 6.2.5) of the isolated DNA (§ 6.2.1) confirmed the mutation had been introduced. Protein was expressed (§ 6.4.2) and the harvested cells were lysed in order to obtain the periplasmic fraction (§ 6.4.5). Ala237Gly was also prepared. The proteins were not purified and were used as crude extracts.

The abilities of Asn132Asp and Ala237Gly to hydrolyse 6-APA [1], Penicillin G [5] and ampicillin [8] were compared by ^1H NMR as follows.

Prior to use in these ^1H NMR experiments, 6-APA [1] was purified by dissolution in water (1 g per 40 mL) using the minimum amount of sodium bicarbonate and re-precipitated by addition of hydrochloric acid to pH 4.5. The resulting precipitate was filtered and dried. It had been found that a small impurity present in commercial sources of 6-APA [1] had an inhibitory effect on class C β -lactamases (Pratt, Dryjanski et al. 1996).

Each substrate was dissolved in 0.1 M phosphate buffer (10 mg mL^{-1}), which was divided into 1 mL aliquots. β -lactamase (100 μL) was then added to the substrate solution. The control aliquot contained no enzyme but had the equivalent volume of phosphate buffer (0.1 M, 100 μL) added. After a period of incubation (1 hour, 37°C , 250rpm) the samples were quenched with methanol (100 μL), filtered through cotton wool then lyophilised. ^1H NMR analysis was carried out in D_2O .

The degree of hydrolysis was calculated by determining the ratios in the ^1H NMR spectrum based on the peaks corresponding to the C-H's of the β -lactam ring. It should also be noted that the resulting hydrolysis products can epimerize and this was taken into account when the ratios were calculated.

Experiments to investigate the hydrolysis of ampicillin [8] with Ala237Gly were carried out. It was found that complete hydrolysis of ampicillin [8] occurred in the presence of wild type β -lactamase, however there was only partial hydrolysis with Ala237Gly ($\sim 80\%$). Partial hydrolysis also occurred with no β -lactamase ($\sim 5\%$).

This result was encouraging. Although previous MIC experiments had shown no difference between wild type and Ala237Gly for 6-APA [1], Penicillin G [5] or ampicillin [8], this showed that the mutation did have some effect on the hydrolysis of Penicillin G [5] albeit small and perhaps in combination with other mutations this could be improved.

Next, the hydrolysis of 6-APA [1], Penicillin G [5] and ampicillin [8] with Asn132Asp was investigated the results of which are summarized in the Table 4.3. It can be seen that compared to wild type, Asn132Asp has a similar ability to hydrolyse 6-APA [1] but a reduced ability for the hydrolysis of Penicillin G [5] and ampicillin [8].

β -lactamase	Substrate					
	6-APA		Penicillin G		Ampicillin	
	SM/%	HP/%	SM/%	HP/%	SM/%	HP/%
Wild type	33	67	23	77	16	84
Asn132Asp	44	56	88	12	89	11
None	90	10	<100	<1	<100	<1

SM Starting material **HP** Hydrolysed Products

Table 4.3 To show the hydrolysis of various substrates with Asn132Asp

These results were promising and it was wondered whether this substrate specific hydrolysis ability could be improved further. Ideally a mutant that can only hydrolyse 6-APA [1] and not IPN [18] was the goal however a mutant with as large a difference in its hydrolysing ability between the two substrates would also be useful. It was decided to combine the Asn132Asp and Ala237Asp mutations and study their combined effects.

4.2.2.7 Generation of Asn132Asp Ala237Gly

The Asn132Asp mutation was introduced into Ala237Gly using site directed mutagenesis (§ 6.3.1. For primers see Appendix D). Subsequent sequencing (§ 6.2.5) of the isolated DNA (§ 6.2.1) confirmed the mutation had been introduced. Protein was expressed (§ 6.4.2) and the harvested cells were lysed in order to obtain the periplasmic fraction (§ 6.4.5). Wild type β -lactamase and the single mutants Asn132Asp and Ala237Gly were also prepared as before.

The hydrolytic capability of the mutants was determined by ^1H NMR as described above and the results are shown in Table 4.4 below. It can be seen that the single mutant Asn132Asp has the greatest difference between its ability to hydrolyse 6-APA [1] and Penicillin G [5] and ampicillin [8]. The single mutant Ala237Gly and the double mutant Asn132Asp Ala237Gly have comparable activities and the difference in their ability to hydrolyse 6-APA [1] over Penicillin G [5] or ampicillin [8] is not as great as that shown for Asn132Asp.

β -lactamase	Substrate					
	6-APA		Penicillin G		Ampicillin	
	SM/%	HP/%	SM/%	HP/%	SM/%	HP/%
Wild type	20	80	46	54	44	56
Asn132Asp	13	87	95	5	91	9
Ala237Gly	68	32	76	24	57	43
Asn132Asp Ala237Gly	51	49	77	23	56	44
None	100	0	100	0	94	6

SM Starting material **HP** Hydrolysed Products

Table 4.4 To show the hydrolysis of various substrates with β -lactamase variants

These ^1H NMR experiments were useful for quantifying the hydrolytic capability of the various mutants compared to wild type β -lactamase. However, it was not known how the mutants would affect the resistance of the corresponding colonies present in any screening or selection method. Therefore, experiments to determine the resistance of the various mutants in XL1-Blue were carried out.

Colonies were streaked onto agar plates containing the various substrates at various concentrations. After a period of incubation (37°C, 18 hours) the surviving colonies were noted. In terms of colony resistance, there was no difference between the single or double mutants. Compared to wild type β -lactamase they had the same resistance to 6-APA [1] more importantly however was the single and double mutants decreased colony resistance to the β -lactams, Penicillin G [5] and ampicillin [8]. The results are shown in Table 4.5 below.

Substrate	Wild type	Asn132Asp	Ala237Gly	Asn132Asp Ala237Gly
10 mM 6-APA	Y	Y	Y	Y
20 mM 6-APA	N	N	N	N
30 mM Pen G	Y	N	N	N
50 mM Pen G	N	N	N	N
60 mM Pen G	N	N	N	N
50 mM Amp	Y	N	N	N

Table 4.5 To show colony survival with various β -lactams

It was also decided to compare the hydrolysis of mixtures of the substrates as in any assay a mixture of acylated and un-acylated 6-APA [1] would be present. This was done for the Asn132Asp mutant only as this mutant so far had given the most promising results.

^1H NMR experiments were set up as before containing 50:50 mixtures of 6-APA [1] plus either Penicillin G [5] or ampicillin [8]. The reactions were followed over one hour with samples taken for analysis by ^1H NMR every ten minutes. Due to the complex mixtures obtained it was necessary to spike the samples with partially hydrolysed substrates in order to correctly identify the relevant peaks in the NMR spectra obtained. For both the Penicillin G [5] and ampicillin [8] experiments, β -lactamase seemed to preferentially hydrolyse the acylated 6-APA over 6-APA [1].

Some example ^1H NMR spectra for the above experiments are shown in Appendix G.

At this point it was decided to use Asn132Asp and Ala237Gly as starting points for the creation of a new library of β -lactamase variants. Although the results so far show that these mutants alone do show improved hydrolysis of 6-APA [1] compared to wild type. However, it was hoped that this could be improved further with additional mutations. Also a mutant with reduced hydrolytic capability for acylated 6-APA [1] was desired so that when both substrates were present 6-APA [1] would be preferentially hydrolysed.

To this end another random library was desired.

4.2.3 Random Library with Rational Mutants

As has been demonstrated random mutation using a mutator strain although a quick way to generate random mutant libraries it was unsuccessful in our case in leading to the identification of suitable β -lactamase mutants. The lack of β -lactamase mutants from using the mutator strain was believed to be due to the fact that mutations were introduced randomly into the entire plasmid and were not restricted to the β -lactamase gene alone. It was desired that mutations would only be present in the gene of interest therefore the use of the commercially available error prone PCR kit GeneMorph® II Random Mutagenesis (Stratagene) was investigated. Another advantage of using this kit is that useful mutation rates with minimal mutational bias are achieved. Also, by varying the amount of DNA used the number of mutations introduced can be controlled. In our case this was important as it was believed only minor mutation of the single mutants Asn132Asp and Ala237Gly was required in order to optimize their selectivity for 6-APA [1] over IPN [18] or other penicillins.

4.2.3.1 Strategy

The strategy for library creation was to carry out error prone PCR according to the supplied protocol (§ 6.3.3). Restriction digest of the resulting PCR product would give the β -lactamase gene fragment, which would then be ligated back into the pUC18 plasmid backbone. The pUC18 backbone would be obtained by digestion using the same restriction enzymes used to obtain the gene fragment, which would allow the backbone and the gene fragment to be ligated without further manipulation.

4.2.3.2 Choice of Restriction Enzymes

Suitable restriction enzymes to isolate both the gene fragment from pUC18 and the pUC18 backbone were selected using Vector NTI® software available from Invitrogen. SspI digests at 186 bp and AlwNI digests at 1468 bp of pUC18. This would result in gene fragments of 1282 bp containing the gene fragment and the backbone of 1404 bp. These fragments are very similar in size, which would make it difficult to excise them from agarose gel after the gel electrophoresis that would be carried out to separate them.

Therefore other enzymes were selected in order to singly digest the undesired fragment in each case.

For the gene fragment *XbaI* was selected. This digests the backbone at 2260 bp. Digestion of the mutagenised pUC18 with *AlwNI*, *XbaI* and *SspI* would result in the desired gene fragment 1282 bp plus two smaller fragments of 792 and 610 bp (Figure 4.4).

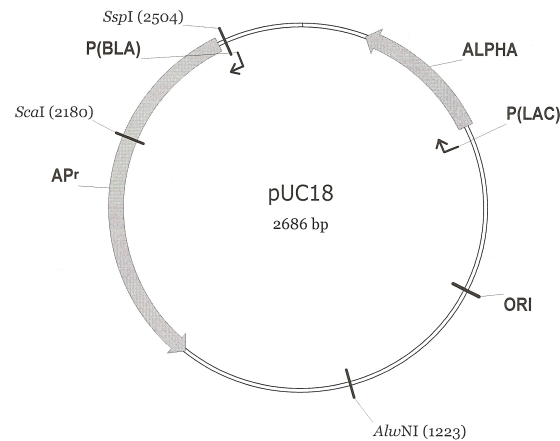


Figure 4.4 Proposed restriction digest of pUC18 to obtain gene fragment

In order to obtain the backbone, *ScaI* would be used which digests the β -lactamase gene at 508 bp. Digestion with *AlwNI*, *SspI* and *ScaI* would result in the desired backbone 1404 bp plus two smaller fragments of 324 and 960 bp. These smaller fragments would be easily separable from the desired fragment in any agarose gel (Figure 4.5)

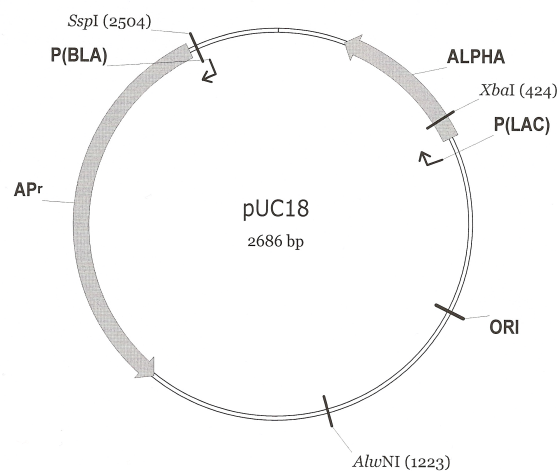


Figure 4.5 Proposed restriction digest of pUC18 to obtain backbone

4.2.3.3 Creation of the Library

Error prone PCR was carried out according to the supplied protocol using a mixture of Asn132Asp, Ala237Gly and Asn132Asp Ala237Gly (20:1:1) pUC18 plasmid was used as the starting point for library generation (for primers see Appendix D). The quantity of DNA added was calculated from Table 4.6. As the β -lactamase gene is 857 bp and the pUC18 plasmid is 2686 bp long, 1550 ng was added in order to obtain a low to medium mutation rate library.

Mutation rate	Mutation frequency (mutations/ kb)	Initial target amount (ng)*	Recommended fold amplification
Low	0-4.5	500-1000	1.5-10
Medium	4.5-9	100-500	10-100
High	9-16	0.1-100	100-10,000

*Not the total amount of template to add but amount of target to amplify.

Table 4.6 To show the quantity of DNA required for various mutation rates

The success of the reaction was determined by quantifying the resulting PCR plasmid product by agarose gel electrophoresis (§). Optimization of the PCR conditions was required and a PCR cycle of 95 °C for 2 minutes; 30 cycles of (95 °C, 1 min; 60 °C, 1 min; 72 °C, 1 min) 72 °C, 10 min; 4 °C, 5 min was eventually employed. These conditions gave a satisfactory yield of PCR product. At this point work ceased due to the completion of this PhD study.

4.3 Chapter 4 Summary

The mutagenesis of β -lactamase RTEM-1 for use in a hydrolysis screen for AT hydrolysis mutants was investigated. A mutant with high selectivity for 6-APA [1] and no selectivity for IPN [18] or other penicillins was desired. A random library of mutants was created using a mutator strain. However subsequent screening of the library did not lead to the identification of any suitable mutants. A rational approach using the X-ray crystal structure of the deacylation-defective mutant Glu166Asn of

RTEM-1 β -lactamase lead to the selection of two residues for mutation, Asn132 and Ala237. Site saturation libraries were created and screened which lead to the identification of the mutant Ala237Gly. Further literature searching revealed the mutant Asn132Asp to also be suitable for our purposes. The selectivity of these mutants and their double Asn132Asp Ala237Gly for 6-APA [1] and other penicillins were studied by ^1H NMR. They had the desired selectivity however we hoped to improve this further in our labs by combining with other random mutations.

CHAPTER FIVE

Equipment & Materials

5.1 Suppliers

The required reagents were generally obtained from Sigma Aldrich® with the following exceptions.

Tryptone was purchased from Oxoid. Glycerol for bacterial stocks was obtained from BDH. Lyophilised DNase I was supplied by Roche. The amylose resin used for MBP affinity chromatography was supplied by New England Biolabs® Inc. (NEB).

SDS-PAGE (§ 6.4.8) reagents (40% acrylamide/Bis solution (37.5:1), *N,N,N',N'*-tetramethyl-ethane-1,2-diamine (TEMED) and ammonium persulfate) were purchased from Bio-Rad. Protein marker, Broad Range (2-212 kDa) was obtained from NEB.

Custom oligonucleotides required for PCR experiments were obtained from Invitrogen. The nucleic acid co-precipitant GlycoBlue™ was purchased from Applied Biosystems.

Our industrial sponsors, DSM, kindly supplied AdiPeN [26], 7-ADCA [11], 7-ACA [4].

5.2 Equipment

Sterilisation of equipment and non-thermolabile solutions was carried out by autoclaving at 121 °C for 20 minutes with a Midas 40 benchtop compact PriorClave.

The incubators used were either a Haraeus Instrument 6000 Incubator, a Jencons –PLS Lab Line Series incubator shaker or a SI 50 Stuart Scientific Orbital incubator. For gentle shaking at RT, an IKA-VIBRAX-VXR orbital shaker was used.

Centrifugation was carried out using a Beckman Microfuge® Lite benchtop Microcentrifuge (<1.5 mL) or a Beckman Allegra™ 21R with a S4180 rotor (up to 50 mL).

UV absorbance measurements were carried out using a Beckman DU® 650 spectrometer.

pH was measured using a Russell RL 150 pH meter.

For PCR an MIC Research Inc., PTC-100™ Programmable Thermal Controller was used.

Milli-Q H₂O was obtained using the Millipore Elix S Milli-Q System. De-ionised water undergoes reverse osmosis *via* Progard™ pack to give water with a resistivity of 18 MW cm.

Gels were visualized on a Bio-Rad Gel DOC 2000 UV Transilluminator.

Proton nuclear magnetic resonance (¹H NMR) spectra and carbon nuclear magnetic resonance (¹³C NMR) spectra were recorded on a Varian Unity INOVA 300E (300 MHz) or a Varian UNITY 500 (500 MHz) NMR spectrometer. Chemical shifts, δ_{H} , are quoted in parts per million (ppm) downfield from tetramethylsilane. Signal splitting patterns are described as singlet (s), doublet (d), triplet (t), quartet (q), quintet (qn), sextet (sx), septet (sp), multiplet (m) or broad singlet (brs). Coupling constants (*J*) are given in Hertz (Hz) to nearest 0.1 Hz.

Fourier Transform Infrared Spectra (FTIR) were recorded on an AT1 Mattson Genesis series FTIR spectrometer, with the sample as a KBr disc, a nujol mull, a film, or a solution in chloroform. Selected absorptions are reported and are quoted in wavenumbers (cm⁻¹).

Low resolution mass spectra were recorded on a Micromass Trio 2000 for electron ionisation and chemical ionisation conditions, and a Micromass Platform for positive and negative ion electrospray analysis.

Melting points were recorded using a Sanyo Gallenkamp variable heater and are quoted to the nearest centigrade.

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5.3 Media/Buffers/Reagents

5.3.1 Microbiological Growth Media

5.3.1.1 2× YT Medium

Per 1 L Milli-Q H₂O:

16 g	Tryptone
10 g	Yeast Extract
5 g	NaCl
(15 g	Bacteriological Agar - <i>for solid media</i>)

Autoclaved at 121 °C for 20 minutes.

5.3.1.2 LB Medium

Per 1 L Milli-Q H₂O:

10 g	Tryptone
5 g	Yeast Extract
10 g	NaCl
(15 g	Bacteriological Agar - <i>for solid media</i>)

Autoclaved at 121 °C for 20 minutes.

5.3.1.3 Glycerol Stocks

Per colony:

750 µL	50:50 Glycerol: Milli-Q H ₂ O (0.22 µm <i>filter sterilized</i>)
750 µL	2× YT supplemented with appropriate antibiotic

Stored at -80°C.

5.3.2 Antibiotic Stock Solutions

5.3.2.1 Chloramphenicol

30 mg Chloramphenicol/mL abs. EtOH

Aliquots (1 mL) stored at -20°C. Added to media/agar at T<50°C. Used at a final concentration of 30 µg mL⁻¹.

5.3.2.2 Ampicillin

50 mg Ampicillin/mL Milli-Q H₂O

Filter sterilized. Single use aliquots (1 mL) stored at -20 °C. Added to media/agar at <50 °C. Used at final concentrations of 50 and 100 mg L⁻¹.

5.3.3 Agarose Gel Electrophoresis Reagents

5.3.3.1 Agarose

Agarose was suspended in 1× TBE then microwaved until molten. The resulting liquid was poured into casts at T<60°C and allowed to cool at RT. See Table 5.1 for agarose concentrations used.

Agarose concentration/%w/v	DNA size/bp
0.5	1,000-30,000
0.7	800-12,000
1.0	500-10,000
1.2	400-7,000
1.5	200-3,000
2.0	50-2,000

Table 5.1 To show agarose concentration required for analysis of DNA

5.3.3.2 1× TBE

90 mM	Tris base
90 mM	Boric acid
2 mM	EDTA
0.5 µg mL ⁻¹	Ethidium bromide

5.3.3.3 6× BJ Agarose Gel Sample Loading Buffer

30%(v/v)	Glycerol
20 mM	EDTA
0.2% (w/v)	Bromophenol Blue

5.3.3.4 1 kb DNA Ladder Solution

The 1kb DNA ladder was purchased from Gibco-BRL and diluted as shown below.

60 µL	1 kb DNA Ladder
167 µL	6× BJ
100 µL	0.2M NaCl
673 µL	Milli-Q H ₂ O

Stored at -20 °C.

5.3.3.5 Restriction Endonuclease Buffers

NEB provides a colour coded 10× NEBuffer with each restriction endonuclease to ensure optimal (100%) activity. (§ Table 5.2 for buffer composition). Some restriction endonucleases require BSA at a final concentration of 100 mg mL⁻¹, which is supplied as a 10 mg mL⁻¹ (100×) stock.

Buffer No.	Colour	Composition
1	Yellow	10 mM Bis Tris Propane-HCl, 10 mM MgCl ₂ , 1 mM DTT, pH 7.0 at 25°C.
2	Blue	50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl ₂ , 1 mM DTT, pH 7.9 at 25°C.
3	Red	100 mM NaCl, 50 mM Tris-HCl, 10 mM MgCl ₂ , 1 mM DTT, pH 7.9 at 25°C.
4	Green	50 mM Potassium acetate, 20 mM Tris-acetate, 10 mM Magnesium acetate, 1 mM DTT, pH 7.9 at 25°C.
BSA Storage Buffer	N/A	20 mM Potassium phosphate, 50 mM NaCl, 0.1 mM EDTA, 5% Glycerol, pH 7.0 at 25°C.

Table 5.2 To show NEBuffer composition

5.3.4 Solutions for the Preparation and Recovery of Competent Cells

5.3.4.1 TFB1

The following stock solutions were prepared:

3M	KOAc	Filter sterilised
1M	CaCl ₂	Autoclaved at 121 °C for 20 minutes
0.5M	MnCl ₂	Autoclaved at 121 °C for 20 minutes
1M	RbCl	Autoclaved at 121 °C for 20 minutes

100 mL buffer contained:

1 mL 3 M KOAc (30 mM final)

1 mL 1 M CaCl₂ (10 mM final)

10 mL 0.5 M MnCl₂ (50 mM final)

10 mL 1 M RbCl (100 mM final)

15 mL Glycerol (15% final)

Adjusted from pH 6.8 to pH 7.4 using 1M acetic acid (100 µL). Filter sterilised and stored at 4 °C.

5.3.4.2 TFB2

The following stock solutions were prepared:

1M KOH

0.75M CaCl₂ Autoclaved at 121 °C for 20 minutes.

1 M MOPS Adjusted from pH 3.3 to pH 6.5 using 1M KOH (16 mL). Filter sterilised and stored at RT.

10 mL buffer contained:

0.1 mL 1M MOPS pH 6.5 (10 mM final)

1 mL 0.75M CaCl₂ (0.75 mM final)

0.1 mL 1M RbCl (10 mM final)

1.5 mL Glycerol (15% final)

Adjusted from pH 6.04 to pH 6.5 using 1M KOH (20 µL). Filter sterilised and stored at 4 °C.

5.3.4.3 SOB

Per 1 L Milli-Q H₂O:

20 g	Tryptone
5 g	Yeast Extract
0.5 g	NaCl

Autoclaved at 121 °C for 20 minutes.

5.3.4.4 SOC

Per 10 mL SOB (§ 5.3.4.3):

200 µL	20%w/v Glucose monohydrate (<i>filter sterilised</i>)
100 µL	1M MgCl ₂ (<i>filter sterilised</i>)
100 µL	1 M MgSO ₄ (<i>filter sterilised</i>)

Prepared and incubated at 37 °C for 1 h immediately before use.

5.3.5 Solutions for the Preparation of Soluble Proteins from *E. coli*

5.3.5.1 Lysis Buffer

Per 10 mL of column buffer (§ 5.3.6.1):

3 mg	EDTA
5 mg	Lyophilized Lysozyme
1 mg	Lyophilized DNase I

Prepared immediately before use.

5.3.5.2 Spheroplast buffer

50 mM Tris Base

5 mM Dithiothreitol

Adjusted to pH 8.0 using dilute HCl_(aq) then sucrose (20%w/v) was added. Prepared immediately before use.

5.3.5.3 TD buffer

50 mM Tris Base

5 mM Dithiothreitol

Adjusted to pH 8.0 using dilute HCl_(aq). Prepared immediately before use.

5.3.6 Solutions for the Purification of Maltose-Binding Protein Fusions

5.3.6.1 Column Buffer

50 mM Tris Base

5 mM Dithiothreitol

Adjusted to pH 7.4 using dilute HCl_(aq). Stored at 4 °C.

5.3.6.2 Elution Buffer

Column buffer (§ 5.3.6.1) containing:

10 mM Maltose

Prepared immediately before use.

5.3.7 Solutions for Protein Analysis

5.3.7.1 Bradford Reagent

0.01% w/v Coomassie Blue G250

5% v/v Ethanol (95%)

8.5% v/v Phosphoric Acid

Filtered. Stored at RT, away from sunlight.

5.3.7.2 HEPES Buffer

50 mM HEPES

Adjusted to pH 7.4 using dilute NaOH_(aq) then sterilized by filtration through a 45 µm membrane. Stored at 4 °C.

5.3.8 SDS-PAGE Buffers and Solutions

5.3.8.1 4× Stacking Buffer

Tris base (60 g, 0.5M) was dissolved in Milli-Q H₂O (800 mL) and the pH adjusted to 6.8 using dilute HCl_(aq). The resulting solution was diluted to 1 L and filtered sterilized through a 0.45 µm membrane. Sodium dodecyl sulphate (4 g, 14 mM) was then added.

Stored at 4 °C.

5.3.8.2 Stacking Gel

For two gels the following quantity was prepared:

3.05 mL Milli-Q H₂O

0.65 mL 40% Acrylamide/Bis solution (37.5:1)

1.25 mL 4× Stacking buffer

37 µL 10% w/v Ammonium persulphate

7 µL TEMED

Used immediately.

5.3.8.3 4× Separating Buffer

Tris base (181.5 g, 1.9 M) is dissolved in Milli-Q H₂O (800 mL) and the pH adjusted to 8.8 using dilute HCl_(aq). The resulting solution is diluted to 1 L and filter sterilized through a 0.45 µm membrane. Sodium dodecyl sulphate (4 g, 14 mM) was then added.

Stored at 4 °C.

5.3.8.4 Separating Gel (10% acrylamide)

For two gels the following quantity was prepared:

6.25 mL	Milli-Q H ₂ O
5.0 mL 40%	Acrylamide/Bis solution (37.5:1)
3.75 mL	4× Separating buffer
5 µL	10% w/v Ammonium persulphate
15 µL	TEMED

Used immediately.

5.3.8.5 5× SDS-PAGE Running Buffer

0.12M	Tris base
0.96M	Glycine
17 mM	Sodium dodecyl sulphate (SDS)

Stored at 4 °C. Diluted with Milli-Q H₂O and used as 1× solution.

5.3.8.6 1× SDS/DTT Loading Buffer

Made using the commercially available (NEB) blue loading buffer pack. The supplied 3× loading buffer and 30× reducing agent are mixed in a 9:1 ratio. For composition see Table 5.3

3× Loading Buffer	187.5 mM Tris-HCl, 6%w/v SDS, 30% Glycerol, 0.03%w/v Bromphenol blue.
30× Reducing Agent	1.25 mM DTT

Table 5.3 To show composition of loading buffer and reducing agent (NEB)

5.3.8.7 Coomassie Blue Stain Solution

0.25 g Coomassie Brilliant Blue R250

90 mL MeOH:H₂O (1:1 v/v)

10 mL Glacial AcOH

Passed through filter paper. Stored at RT in a brown bottle. Reusable.

5.3.8.8 Coomassie Blue Destain Solution

500 mL Ethanol

200 mL Glacial AcOH

Diluted to 2 L with H₂O. Stored at RT.

CHAPTER SIX

General Techniques

6.1 General Information

Unless stated otherwise the reagents and procedures detailed below are based on those contained in *Molecular Cloning, A Laboratory Manual*, 3rd ed, by Sambrook J. and Russell D. W.

6.2 DNA Preparation and Analysis

6.2.1 DNA Isolation Using QIAGEN's QIAprep® Miniprep Kit

Glycerol stocks were streaked onto 2× YT agar (1.5%) plates supplemented with the appropriate antibiotic and incubated (37 °C, 24 hours). A single colony was tooth-picked into 2× YT (5 mL) supplemented with the appropriate antibiotic and incubated (27 °C, 24 hours). The resulting cell culture (3 mL) was centrifuged (14000rpm, RT, 5 min).

The DNA was isolated and purified from the resulting cell pellet using QIAGEN's QIAprep® Miniprep kit according to the supplied protocol. The procedure is based on the alkaline lysis of bacterial cells. The DNA in the resulting lysate is selectively adsorbed onto the silica membrane of the supplied columns in the presence of a high-salt buffer, followed by elution with a low-salt buffer or H₂O. This procedure typically yielded ~35-45 µL of 150 ng µL⁻¹ DNA solution. The resulting DNA solution was stored at -20 °C.

6.2.2 Quantification of DNA by Absorbance at OD₂₆₀

The concentration of DNA can be determined by absorbance at 260 nm. However, only purified samples can be quantified in this way as impurities, including DNA, RNA, EDTA and phenol, can absorb at this wavelength hence greatly affect the reading.

An OD₂₆₀ reading of 1 corresponds to ~33 µg mL⁻¹ of single stranded oligonucleotide, and ~40 µg mL⁻¹ and ~50 µg mL⁻¹ of ssDNA and dsDNA respectively.

The OD₂₆₀:OD₂₈₀ ratio indicated the purity of the DNA sample, with values of 1.8 indicating pure samples. Samples with values less than this were re-purified.

DNA samples were diluted in Milli-Q H₂O (1:20) and their OD₂₆₀ determined using a quartz cuvette. Milli-Q H₂O was used as a blank.

6.2.3 Quantification of DNA by Agarose Gel Electrophoresis

Horizontal agarose gel electrophoresis was performed using Mini-Sub cell GT electrophoresis tanks (Bio-Rad). Gels were cast according to the manufacturer's (Bio-Rad) instructions as follows. Agarose (§ 5.3.3.1 for concentrations used) was microwaved until molten then allowed to cool to T<60 °C before pouring into the cast. The gel was allowed to set at RT for approximately one hour. The gel assembly was then placed in the electrophoresis tank and 1× TBE buffer added to the tank. 6× BJ (4 µL) was added to the DNA samples, which were then loaded into the wells using a pipette. The DNA samples were linearised by restriction endonuclease digest (§ 6.2.4) prior to electrophoresis. A range of quantities of 1.1kb DNA standard (20 ng µL⁻¹, Stratagene) with 6× BJ (4 µL) were loaded. A DNA ladder (20 µL, 60 ng µL⁻¹) was also loaded. The tank was then connected to a power pack and a voltage applied to the gel. Gels were typically run at 100V for 20 min or until the blue dye reached the front of the gel. Gels were visualised using a UV Transilluminator - UV light setting. The quantity of DNA in the sample(s) was determined by comparison with the DNA standard.

6.2.4 DNA Characterisation by Restriction Endonuclease Digest

Diagnostic cuts of DNA were carried out using restriction endonucleases supplied by NEB. Double digests were also carried out; the compatibility of the endonucleases and appropriate buffer were verified online at www.neb.com. Reaction mixtures typically contained the following:

- 3 µL DNA (~100 ng µL⁻¹)
- 2 µL 10× Buffer (supplied with enzyme)
- 0.5 µL Enzyme
- 15 µL Milli-Q H₂O

The components were mixed using a pipette prior to addition of the enzyme. The resulting mixture was incubated (37 °C, 1 h). 6× BJ (4 µL) was added to the reaction mixture prior to analysis by agarose gel electrophoresis (§ 6.2.3) (all 24 µL was loaded onto the gel). A DNA ladder (20 µL, 60 ng µL⁻¹) was also loaded. The gels were run at 50V for 90 min. These conditions allowed optimal resolution of the DNA bands in the ladder enabling comparison with those of the DNA sample.

6.2.5 DNA Sequence Determination

Samples were prepared for DNA sequencing using the commercially available BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) as follows.

Reaction mixtures were prepared containing:

4 µL DNA (100 ng µL⁻¹)

1 µL BigDye® v3.1

3.5 µL 5× Sequencing Buffer

2 µL Primer – sense/antisense (diluted to 20 µM with elution buffer)

9.5 µL Milli-Q H₂O

The samples were mixed with a pipette before placing on a thermal cycler. A program of [96 °C, 2 min; 30× (96 °C, 40 s; 50 °C, 15 s; 60 °C, 4 min); 4 °C, 5 min] was used. The resulting PCR products were purified by ethanol precipitation (§ 6.2.6). Sequencing was performed using an ABI 377 automated sequencer or an ABI 3100 sequencer. The results were analysed using Vector NTI Advance™ software.

6.2.6 Purification of DNA by Ethanol Precipitation

DNA solutions were added to a mixture of the following:

2 μ L 3M NaOAc (aq.) pH 5.2

2 μ L 125 mM EDTA (aq.) pH 8.0

50 μ L EtOH (absolute)

GlycoBlue™ (0.5 μ L) was also added as a co-precipitant during the preparation of samples for DNA sequencing (§ 6.2.5). The resulting mixture was left to stand at RT for 10 min, then centrifuged (14000rpm, RT, 30 min) and the supernatant carefully decanted using a pipette. Ethanol (70%, 250 μ L) was added and the sample centrifuged (14000rpm, RT, 10 min). After decanting the supernatant from the sample, more ethanol (70%, 250 μ L) was added and the sample again centrifuged (14000rpm, RT, 10 min). The ethanol was carefully decanted and any remaining ethanol traces allowed to evaporate at RT overnight.

6.2.7 Purification of DNA Using QIAGEN's QIAquick® Gel Extraction Kit

DNA fragments from endonuclease digests (§ 6.2.4) were excised from agarose gels (§ 6.2.3) using a scalpel and purified using QIAGEN's QIAquick® Gel Extraction kit according to the supplied protocol. The procedure relies on the use of buffers, which selectively adsorb and elute DNA, at high and low salt concentrations respectively, to and from the silica membrane of the supplied column.

6.3 Mutagenesis Techniques

6.3.1 Site Directed Mutagenesis

Site directed mutagenesis was performed using Stratagene's QuickChange® Site Directed Mutagenesis kit according to the supplied protocol. PCR mixtures were set up as follows:

1 μL dsDNA template ($50 \text{ ng } \mu\text{L}^{-1}$)

3 μL Forward primer ($40 \text{ ng } \mu\text{L}^{-1}$)

3 μL Reverse primer ($40 \text{ ng } \mu\text{L}^{-1}$)

5 μL 10 \times Buffer

1 μL dNTP mix

37 μL Milli-Q H₂O

Pfu Turbo DNA polymerase ($2.5 \text{ U } \mu\text{L}^{-1}$, 1 μL) was added and the reaction mixed with a pipette. A PCR program of [95 °C, 30 s; 16 \times (95 °C, 30 s; 55 °C, 1 min; 68 °C, 13 min 15 s); 4 °C, 5 min] was used.

Dpn I ($10 \text{ U } \mu\text{L}^{-1}$, 1 μL) was then added directly to the amplified reaction mixture, which was then mixed thoroughly using a pipette before incubating (37 °C, 1 h) in order to digest the parental DNA (*i.e.* methylated, non-mutated). The degree of amplification was verified by agarose gel electrophoresis (§ 6.2.3) after the *Dpn* I digest.

The resulting PCR mixture could then be transformed (§ 6.3.4 or § 6.3.5) directly. However, the success rate of transformation was greatly improved by prior purification of the DNA by ethanol precipitation (§ 6.2.6) and re-suspension of the DNA in Milli-Q H₂O.

6.3.2 Random Mutagenesis - using a Mutator Strain (XL1-Red)

Random mutagenesis was performed using the commercially available mutator strain, XL1-Red (Stratagene), with slight modification to the supplied protocol as follows.

The supplied XL1-Red competent cells were thawed on ice and 100 μ L aliquots pipetted into 14 mL Falcon polypropylene tubes. 1.42M β -mercaptoethanol (1.7 μ L) was added to give a final concentration of 25 mM and the resulting mixture kept on ice for 10 min with occasional swirling.

DNA (1 μ L, 10-50 ng) was added and the mixture kept on ice for 30 min. The tubes were then heat pulsed (42 °C, 45 s) in a water bath then placed back onto ice for 2 min. Pre-warmed (42 °C) SOC (900 μ L) was added then the solution was incubated (37 °C, 1 h, 250rpm).

The inoculum was centrifuged (15000rpm, RT, 5 min) and the resulting cell pellet re-suspended in SOC and plated onto LB agar plates containing appropriate antibiotic. The plates were then incubated (37 °C, 24 h).

The colonies were washed off the agar plate using LB media to give a stock solution, which could be used to generate inoculated media. Subsequent DNA isolation (§ 6.2.1) and transformation (§ 6.3.4 or § 6.3.5) into XL1-Blue allowed screening of the mutants as desired.

6.3.3 Random Mutagenesis – using Error Prone PCR

Error prone PCR (epPCR) was performed using Stratagene's GeneMorph II Random Mutagenesis kit according to the supplied protocol. PCR mixtures were set up as follows:

1 μL DNA template (see Table 6.1 for amount)

0.5 μL Primer mix (250 ng μL^{-1} of each primer)

5 μL 10 \times Mutazyme II Reaction Buffer

1 μL 40 mM dNTP mix (200 μM each final)

1 μL Mutazyme II DNA polymerase (2.5U μL^{-1})

41.5 μL Milli-Q H_2O

Mutation rate	Mutation frequency (mutations/ kb)	Initial target amount (ng)*	Recommended fold amplification
Low	0-4.5	500-1000	1.5-10
Medium	4.5-9	100-500	10-100
High	9-16	0.1-100	100-10,000

*Not the total amount of template to add but amount of target to amplify.

Table 6.1 To show the quantity of DNA required for various mutation rates

Components were mixed using a pipette before addition of the Mutazyme II DNA polymerase. The mixture was centrifuged briefly before undergoing the following PCR program [95 °C, 2 min; 30 \times (95 °C, 1 min; 60 °C, 1 min; 72 °C, 1 min); 72 °C, 10 min; 4 °C, 5 min].

The PCR product yield was quantified by comparison with the supplied 1.1-kb standard by agarose gel electrophoresis (§ 6.2.3).

6.3.4 Transformation – Heat Shock Method

Transformation was carried out using XL1-Blue supercompetent cells (Stratagene) as follows.

DNA (1 μ L) was added to thawed XL1-Blue supercompetent cells (40 μ L), mixed thoroughly with a pipette, then kept on ice for 30 min. The tubes were then heat pulsed (42 °C, 45 s) in a water bath then placed back onto ice for 2 min. Pre-warmed (37 °C) SOC (960 μ L) was added then the tube was incubated (37 °C, 2 h, 250rpm).

800 μ L of the cell suspension was centrifuged (14000rpm, RT, 5 min) and the resulting cell pellet re-suspended in fresh, SOC media (50 μ L) and streaked onto 2 \times YT agar plates supplemented with the appropriate antibiotic. The plates were then incubated (37 °C, 18 h) to allow the transformed colonies to develop.

6.3.5 Transformation - Electroporation Method

Electrocompetent cells (40 μ L) (§) were thawed on ice and 1.42 mM β -mercaptoethanol (0.68 μ L) was added to a final concentration of 25 mM and the resulting mixture kept on ice for 10 min with occasional swirling.

DNA (1 μ L, 0.1-200 ng) was added and mixed using a pipette. It was important that the DNA had a low ionic concentration to prevent arcing between the electrodes of the cuvette during the electroporation process. The mixture (50 μ L) was added to a chilled cuvette (Gene Pulser®, 0.1 cm electrode gap, Bio-Rad) ensuring there were no air bubbles present.

The cuvette was placed in the electroporation chamber (*E. coli* Pulser Electroporation apparatus, Bio-Rad) and subjected to a single pulse of 1.7 kV at a capacitance setting of 25 μ F and a resistance of 200 Ω . These conditions correspond to a field strength of 8.5 kV cm⁻¹ with a time constant between 3.6 and 4.0 ms.

The cells were then added to pre-warmed (37 °C) SOC (960 μ L) then incubated (37 °C, 2 h, 250rpm). 800 μ L of the cell suspension was centrifuged (14000rpm, RT, 5 min) and the resulting cell pellet re-suspended in fresh, SOC media (50 μ L) and streaked

onto 2× YT agar plates supplemented with the appropriate antibiotic. The plates were incubated (37 °C, 18 h) to allow the transformed colonies to develop.

6.3.6 Preparation of Competent Cells for Electroporation

Glycerol freezes were thawed and streaked onto LB agar plates supplemented with the appropriate antibiotic. After incubation (37 °C, 24 h) a single colony was tooth picked into LB (5 mL) and incubated (37 °C, 24 h). 3 ml of the resulting cell culture was added to LB (250 mL) plus 0.1M MgSO₄ (50 mL) and the resulting 1% inoculum incubated (37 °C, 180rpm) until the OD₆₀₀ measured between 0.4 and 0.6 (approximately 3 h). The cell suspension was cooled on ice for 10 min then harvested by centrifugation (5500rpm, 4 °C, 5 min).

The cell pellets were re-combined using ice cold TFB1 (100 mL) and the resulting suspension kept on ice for 10 min. After centrifugation (5500rpm, 4 °C, 5 min) the cell pellets were re-combined using ice cold TFB2 (10 mL) and the resulting suspension kept on ice for 30 min. Aliquots (200 µL) were snap frozen using a dry ice/acetone bath then stored immediately at -80 °C.

6.4 Protein Preparation

6.4.1 AT Protein Expression

Glycerol freezes were thawed and streaked onto 2× YT agar plates supplemented with chloramphenicol (30 µg mL⁻¹). After incubation (27 °C, 48 h) a single colony was tooth picked into 2× YT (5 mL) supplemented with chloramphenicol (30 µg mL⁻¹) and incubated (27 °C, 24 h). 1 ml of the resulting cell culture was added to 2× YT (100 mL) supplemented with chloramphenicol (30 µg mL⁻¹) and the resulting 1% inoculum incubated (27 °C 250rpm) until the OD₆₀₀ measured >0.4 (usually 5 -6 hours). The cells were induced with IPTG (0.1M, 300 µL) and incubated (27 °C, 16 h, 250rpm).

The induced cells were harvested by centrifuging (5500rpm, 4 °C, 30 min). The supernatant was discarded and the resulting cell pellet stored at -80 °C for at least 1 hour, usually overnight, prior to lysis (§ 6.4.3, § 6.4.4 or § 6.4.5).

6.4.2 β – Lactamase Protein Expression

Glycerol freezes were thawed and streaked onto 2× YT agar plates supplemented with ampicillin (50 mg L⁻¹). After incubation (37 °C, 16 h) a single colony was tooth picked into 2× YT (5 mL) supplemented with ampicillin (50 mg L⁻¹) and incubated (27 °C, 24 h). 1 ml of the resulting cell culture was added to 2× YT (100 mL) supplemented with ampicillin (50 mg L⁻¹) and the resulting 1% inoculum incubated (37 °C, 16 h, 250rpm).

The cells were harvested by centrifuging (5500rpm, 4 °C, 30 min). The supernatant was discarded and the resulting cell pellet stored at -80 °C for at least 1 hour, usually overnight, prior to lysis (§ 6.4.5).

6.4.3 *E. coli* Cell Lysis – Sonication Method

Cells underwent pulse sonication using a Sanyo MSE Soniprep 150 machine as follows.

Cell pellets were thawed on ice, re-suspended in lysis buffer (5 mL) then kept on ice for 1 hour. The lysate then underwent pulse sonication, which consisted of 10 cycles of 10 s at 10 μ m amplitude followed by 20 s rest. The lysate was kept on ice throughout sonication. Protamine sulphate (5% w/v) was added and the resulting suspension centrifuged (5500rpm, 4 °C, 30 min). The supernatant was transferred to a clean falcon tube and stored at -80 °C until required.

6.4.4 *E. coli* Cell Lysis – BugBuster® Protein Extraction Reagent

Cell lysis was performed using Novagen®'s BugBuster® Protein Extraction Reagent according to the supplied protocol as follows.

The cell pellet was thawed on ice then re-suspended in the BugBuster® reagent (5 ml per g of cells). The resulting solution was incubated (24 °C, 30 min, 100rpm), and then centrifuged (5500rpm, 4 °C, 30 min). The supernatant was transferred to a clean falcon tube and stored at -80 °C.

6.4.5 Fractionation of *E. coli* to obtain Periplasmic and Cytoplasmic Protein

The cell pellets were thawed on ice and re-suspended in spheroplast buffer (10 mL per cell pellet from 50 mL culture). The resulting solution was aliquoted (1.4 mL) and lysozyme (2 mg mL⁻¹ stock solution) was added to a final concentration of 0.1 mg mL⁻¹. The sample was inverted to mix then incubated (37 °C, 20 min). After addition of 0.5M EDTA (30 µL), the sample was inverted to mix then centrifuged (13000rpm, RT, 90 s). 750 µL from the top of the supernatant was removed to give the periplasmic fraction.

The remainder of the supernatant was removed and the cell pellet re-suspended in TD buffer (1 mL). The solution was centrifuged (13000rpm, RT, 2 min) and the resulting supernatant was the cytoplasmic fraction.

6.4.6 Purification of MBP Fusions by Affinity Chromatography

Ideally, protein purification is carried out in a cold room (4 °C). However, the procedure was routinely carried out at RT, using ice-cold buffers and keeping all materials on ice throughout, with no apparent loss of yield or activity.

The columns were prepared, as follows, using a 10 mL syringe with the plunger removed as the column. Glass wool was used to plug the 'column', which was then packed with amylose resin (5 mL). The resin was washed with column buffer (40 mL) to equilibrate the resin.

The cell free extract was thawed on ice and column buffer (25 mL) added. The resulting solution was applied to the column. The eluted material was collected and applied to the column to ensure complete saturation of the resin. The resin was washed with column buffer (40 mL) and the eluent monitored using Bradford reagent (10 µL eluent plus 100 µL Bradford reagent). The eluent initially tests positive for protein however, towards the end of the wash protein should no longer be evident.

The desired, maltose binding protein-protein fusion was eluted from the resin, using elution buffer (10 mL), and collected in fractions (1 mL). The fractions were analysed with Bradford reagent and those containing protein were combined and aliquoted (200-500 µL aliquots). The protein aliquots were stored at -80 °C until required.

6.4.7 Quantification of Protein by Bradford Assay

Proteins were quantified using the colorimetric Bradford Assay method (Bradford 1976), as follows:

Standard solutions ($1\ \mu\text{g}\ \mu\text{L}^{-1}$ to $0.25\ \mu\text{g}\ \mu\text{L}^{-1}$) of bovine serum albumin (BSA) in column buffer were freshly prepared. Each standard solution ($10\ \mu\text{L}$) was added to Bradford reagent ($1\ \text{mL}$) and the absorbance at $595\ \text{nm}$ measured. Column buffer was used as a blank. A calibration plot was obtained.

The protein sample solutions ($10\ \mu\text{L}$) were also added to Bradford reagent ($1\ \text{mL}$) and the absorbance at $595\ \text{nm}$ measured. The concentration of protein in the sample was determined by reference to the calibration plot.

6.4.8 Analysis of Protein by SDS - PAGE

Proteins were characterized using SDS-PAGE (Laemmli 1970) as follows. Electrophoresis was carried out in a Mini Protean II electrophoresis chamber (Bio-Rad). (Laemmli 1970) (Laemmli 1970) (Laemmli 1970)

Gels ($0.75\ \text{mm}$) were cast according to the manufacturer's (Bio-Rad) instructions as follows. The cast was set up as described in the instructions. Separating gel was prepared and immediately poured in to $1\ \text{cm}$ below the bottom of the wells, using the comb as a guide. The gel was covered with water-saturated *iso*-butanol and incubated ($37\ ^\circ\text{C}$, $1\ \text{h}$). The *iso*-butanol was carefully rinsed off the top of the gel using Milli-Q H_2O or separating buffer and the comb was placed in the top of the cast. The stacking gel was prepared and immediately poured into the cast until it reached the level of the top of the wells. After incubation ($37\ ^\circ\text{C}$, $1\ \text{h}$) the comb was removed and the wells were rinsed with Milli-Q H_2O or running buffer. The gel was then removed from the cast and placed into the electrophoresis tank. $1\times$ SDS running buffer was poured into the tank, completely covering the gel, and the whole assembly placed in an ice bath.

The samples ($20\ \mu\text{L}$) were prepared by adding $1\times$ SDS/DTT ($10\ \mu\text{L}$). The samples and protein marker were denatured ($95\ ^\circ\text{C}$, $5\ \text{min}$) prior to loading into the wells of the gel. The samples ($15\ \mu\text{L}$) and protein marker ($15\ \mu\text{L}$) were loaded using a pipette with gel

saver tips. 1× SDS/DTT (15 µL) was added to any unused wells to ensure the gel ran evenly and without ‘smiling’.

The tank was then connected to a power pack and a voltage applied to the gel. Gels were typically run at 20 mA for a single gel or 40 mA for two gels, until the bromophenol blue dye had reached the front of the gel, typically 90–120 min. The power was then carefully switched off and the gel removed from the apparatus in preparation for staining.

The gel was stained by covering with Coomassie Blue Stain solution and microwaving for 20 s followed by shaking (100rpm, RT, 2 min). The Coomassie Blue Stain solution (reusable) was decanted off and the gel covered with Coomassie Blue destain solution, microwaved for 20 s then shaken (100rpm, RT, 2 min). Staining and destaining was carried out twice. Finally, the gel was covered with destain and left to shake overnight (100rpm, RT). The destain was then poured off and the gel visualised using a UV Transilluminator - EPI light setting.

6.5 Acylation Screening

6.5.1 Amylose Affinity Membrane Screen Protocol

The procedure used to screen AT mutant libraries for improved acylation activity was developed by previous group members, Cheung (2006) and Khara (2007) and was carried out as follows.

The library glycerol stock was diluted and streaked onto 2× YT agar plates supplemented with chloramphenicol (30 µg mL⁻¹) and IPTG (0.3 mM) and incubated (27 °C, 48 h). The library was diluted to give approximately 1500 – 2000 colonies per 22.5 × 22.5 cm plate.

The colonies were then ‘lifted’ using an amylose affinity membrane (§ 6.5.2). The membranes containing the bound colonies underwent a freeze-thaw cycle (-80 °C, 30 min; 27 °C, 30 min), twice. The membranes were then placed, cells facing upwards, on top of filter papers that had been pre-soaked in lysis buffer and sprayed with more lysis buffer. After incubation (27 °C, 90 min), the membranes were washed by placing in column buffer (150 mL) and shaking (200rpm, RT, 30 min). The membranes were

then dried, by placing on paper towels, before being placed, cells facing downwards, onto 2× YT agar plates containing *ESS E. coli* (§ 6.6.1).

Depending on the solubility of the substrates being screened, they were either added to the *ESS E. coli* agar plates during their preparation, or, sprayed as an aqueous solution with a small amount of organic solvent (*e.g.* THF 5% v/v) on to the membranes once they had been placed on top of the *ESS E. coli* agar plates. Substrates were used at such concentrations that if acylation occurred the size of kill zone produced by an individual colony was large enough to visualise but not so large as to mask the effects of surrounding colonies.

The *ESS E. coli* plate-membrane sandwich was then incubated (27 °C, 1 h) after which time the membranes were removed and the *ESS E. coli* agar plates incubated (37 °C, 18 h). The plates were checked by eye, for kill zones and the responsible colonies were selected from the original master plate for further investigation.

6.5.2 Amylose Affinity Membrane Preparation

The preparation of the membranes required for the acylation screening (§ 6.5.1) was based on work by Cattoli and Sarti (Cattoli and Sarti 2002).

Cellulose matrix membranes (Whatman® 541 Hardened Ashless Cellulose filter paper 24 cm diameter) (×50) were placed in an aqueous solution of sodium hydroxide (0.6M, 1.1 L) containing sodium borohydride (2.2 g) and 1,4-diglycidyl ether (100%, 180 mL), and shaken (200rpm, RT, 8 h). The filter papers were washed twice, by placing in Milli-Q H₂O and shaking (200rpm, RT, 5 min), then placed in an aqueous solution of sodium hydroxide (0.6M, 320 mL) containing sodium borohydride (640 mg) and amylose solution (50 mg mL⁻¹, 320 mL), and shaking (200rpm, RT, 18 h).

The filter papers were washed by shaking (200rpm, RT, 10 min) in a solution of LDAO (0.1M, 1 L), and then placed in 2-ethanolamine (1M, 400 mL) and shaken, (200rpm, RT, 24 h).

The filter papers were finally washed by placing alternately, in an aqueous solution of sodium chloride (1M, 800 mL) and shaking (200rpm, RT, 20 min) then, water (800 mL)

and shaking (200rpm, RT, 20 min), twice. The filter papers were then dried on paper towels before being wrapped in foil and sterilised by autoclaving at 121 °C for 20 min.

The resulting membranes were stored at 4 °C and sterilised by UV irradiation before use.

6.6 AT Mutant Activity Determination

Kinetic studies were routinely carried out, in triplicate, in order to compare the activity of newly identified mutants with wild type AT and other mutants. Due to the comparative nature, it was essential that all the proteins used in any one kinetic study were expressed and purified together, as a batch, using the same reagents and buffers to ensure the test was, as far as possible, fair.

Generally, reactions were set up containing a β -lactam nucleus and acyl donor plus AT and the reactions incubated. Samples were taken at various time points and were quenched by the addition of ice-cold methanol. Substrate concentrations were such that upon addition of samples to a bioplate, measurable kill zones would be obtained.

6.6.1 *ESS E. coli* Bioplate Preparation

ESS E. coli was kindly provided by DSM, Delft, The Netherlands. Single colonies of *ESS E. coli* were tooth picked into 2× YT (5 mL) and incubated (37 °C, 18 h, 250rpm). The resulting cell culture was diluted using glycerol:Milli-Q H₂O in a 1:1 ratio, then aliquoted (300 μ L). The resulting glycerol stocks were stored at -80 °C until required.

ESS E. coli glycerol stock (300 μ L) was added to 2× YT (100 mL) and incubated (27 °C, 250rpm) until the OD₆₀₀ absorbance measured 2.0 (approximately 12 h). The cell culture was then diluted ten-fold using freshly prepared 2× YT agar at T<42 °C to give a final OD₆₀₀ absorbance of 0.2. The mixture was then poured into Petri dishes (18 mL per round Petri dish (90 mm), or 70 mL per square Petri dish (22.5 × 22.5 cm)). The plates were allowed to cool at RT for 30 min. The plates were stored at 4 °C and used within two days. Prior to use the plates were allowed to equilibrate at RT for

30 min then a cork borer was used to make wells (7 mm diameter) in the plates immediately before use.

6.6.2 Calibration of *ESS E. coli* Bio Plates

Each batch of *ESS E. coli* bio plates was calibrated with respect to AdiPen [26] as follows.

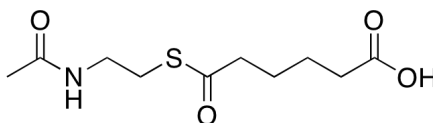
A range of AdiPen [26] standards, (3.2 – 64 nM in 40 μ L) were prepared by serial dilution of a stock solution (1.6 mM) using HEPES buffer and were pipetted into the wells of bioplates. Any plate-to-plate variations were accounted for by placing duplicate aliquots on different plates. The plates were then incubated (37 °C, 18 h) and the resulting kill zones measured in mm. Kill zones were the diameter of the clear ring around the well minus the diameter of the well and correspond to growth inhibition due to diffusion of the antibiotic into the agar. Calibration curves could then be plotted from the resulting data.

6.6.3 Bioplate Assay (Fleming 1989)

6-APA [1] (1.6 mM, 100 μ L), AdiCoA [32] or AdiNAC [33] (5 mM, 100 μ L) were mixed together and pre-warmed by placing in a heating block (26°C, 5 min). A solution of the enzyme, made up to 200 μ L in HEPES buffer (50 mM, pH 7.4) with DTT (5 mM) was then added. The resulting mixture was mixed using a pipette. Aliquots (40 μ L) were taken every 10 min. The aliquots were quenched by adding to ice-cold methanol (40 μ L), vortexing then placing on ice. The aliquot (80 μ L) was added to the well of a bioplate, which was then incubated (37°C, 18 h). The kill zones were measured in mm and the amount of conversion determined by reference to the calibration curves (§ 6.6.2).

6.7 Synthesis of Substrates

6.7.1 Preparation of *S*-Adipoyl-*N*-Acetyl-Cysteamine (AdiNAC) [33]

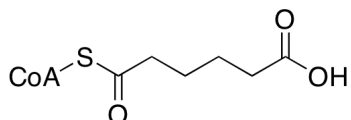


Under anhydrous conditions, *N*-acetylcysteamine (1.78 mL, 16.8 mM) and triethylamine (2.34 mL, 16.8 mM) in THF (50 mL) were added drop wise over 30 minutes to a solution of adipoyl chloride (2.44 mL, 16.8 mM) in THF (50 mL) then left to stir at room temperature for 1 hour. The resulting suspension was filtered by suction to remove the precipitated Et₃N·HCl salt, and the solution concentrated *in vacuo* to give an amber oil. This oil was dissolved in H₂O and extracted with EtOAc (2 × 50 mL). The organic extracts were combined and the volume reduced by half *in vacuo*. A saturated solution of sodium bicarbonate was added until the pH was 5.5 and the organic layer was back-extracted with H₂O (2 × 50 mL). The aqueous extracts were combined and the H₂O removed by lyophilisation to give the title compound as a white solid (1.67 g, 38%).

m.p. 235-237°C; ν_{max} (film)/cm⁻¹ 3292m (NH), 1692s (COOH), 1640s (NHCO), 1562s (SCO); δ_{H} (CDCl₃, 300 MHz) 1.39-1.57 (4H, m, CH₂CH₂CH₂CO), 1.82 (3H, s, CH₃), 2.09 (2H, t, *J* 7.57, CH₂CO₂H), 2.56 (2H, t, *J* 6.94, SCOCH₂), 2.95 (2H, t, *J* 6.15,

SCH₂), 3.26 (2H, t, *J* 6.30, HNCH₂); *m/z* (Cl⁺) 248 (M-H 20%), calculated for C₁₀H₁₇O₄NS = 246.0878.

6.7.2 Preparation of Adipoyl Coenzyme A (AdiCoA) [32]



Coenzyme A sodium salt (100 mg, 1.3×10^{-4} mol) was suspended in a mixture of acetone (15 mL) and H₂O (150 μ L). The mixture was cooled by placing in an ice-bath and the pH adjusted to 8 using 0.2 M NaHCO₃. Adipoyl chloride (37.98 μ L, 2.6×10^{-4} mol) was added slowly dropwise to the stirring solution. After one hour of stirring on ice and careful maintenance of the pH, the reaction was concentrated *in vacuo* then lyophilised to give the title compound as a white solid (113.4 mg, 95%).

δ_H (D₂O, 500 MHz) 0.59 (3H, s, CH₃CCH₃), 0.74 (3H, s, CH₃CCH₃), 1.36-1.54 (4H, m, CH₂CH₂CH₂CO), 2.08 (2H, t, *J* 6.78, NHCOCH₂CH₂), 2.30 (2H, t, *J* 6.47, CH₂CO₂H), 2.34 (2H, t, *J* 6.62, SCOCH₂), 2.62 (2H, t, *J* 6.31, SCH₂CH₂NH), 3.19 (2H, t, *J* 6.47, SCH₂CH₂NH/COCH₂CH₂NH), 3.31 (2H, t, *J* 5.05 SCH₂CH₂NH/COCH₂CH₂NH), 3.38-3.46 (1H, m, OCHCH₂HOPO₃⁻/OCHCH₂OH), 3.67-3.74 (1H, m, OCHCH₂HOPO₃⁻/OCHCH₂OH), 3.87 (1H, s, COCH₂OH), 4.10 (2H, s, (CH₃)₂CCH₂), 4.46 (1H, s, OCHCH₂HOPO₃⁻), 6.05 (1H, d, *J* 6.94, OCHCH₂OH), 8.13 (1H, s, NCHN/ NH₂CNCH), 8.41 (1H, s, NCHN/ NH₂CNCH)

m/z (ES⁻) 894.2 (M-H 4.7%), 187.4 (100%), 218.4 (64%), calculated for C₂₇H₄₄O₁₉N₇SP₃ = 895.6615.

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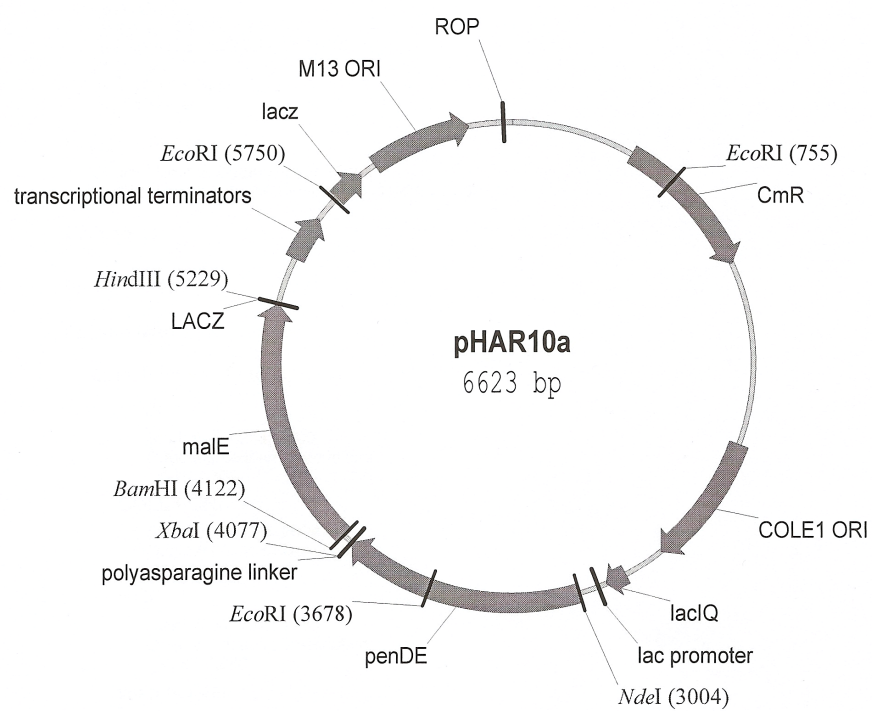
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APPENDICES

Appendix A pHAR10a Plasmid Map



Appendix B pHAR4-p Plasmid Map

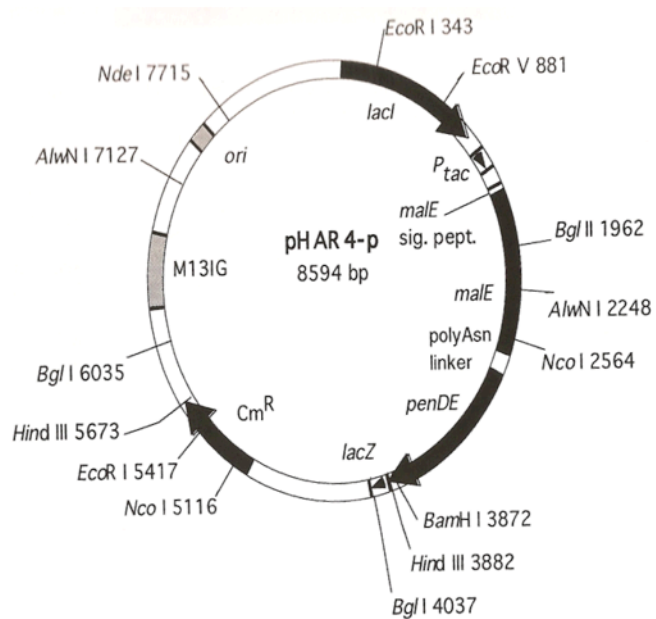
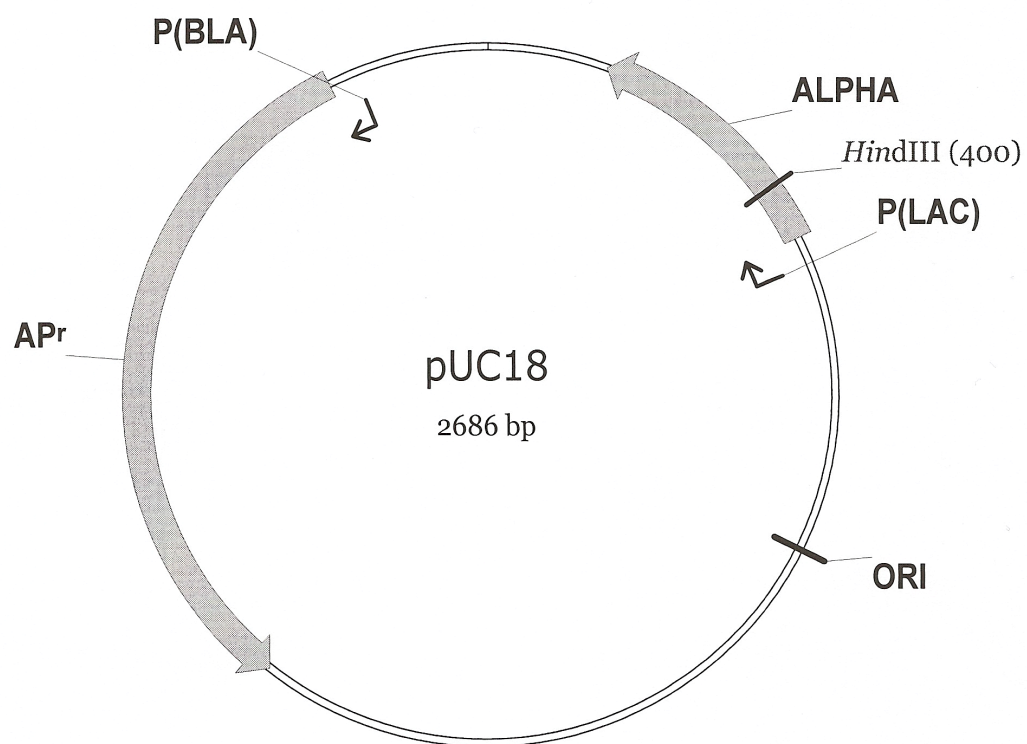


Image taken from Darren Hart's thesis

Appendix C pUC18 Plasmid Map



Appendix D Oligonucleotide Sequences

Chapter 2 pHAR10a plasmid (6623bp) *penDE* (3005-4075)

Sequencing Primers

	2901	2921
S0	5'-TTA GCT CAC TCA TTA GGC ACC-3'	
	2998	3024
S1	5'-AGA CCA TAT GCT TCA CAT CCT CTG TCA-3'	
	3251	3271
S2	5'-ATT GTC ATG CTT AAT ACC CGC-3'	
	3271	3251
AS2	5'-GCG GGT ATT AAG CAT GAC AAT-3'	
	3311	3331
S3	5'-TGC ACC ACT GCC TAT TGT CAA-3'	
	3331	3311
AS3	5'-TTG ACA ATA GGC AGT GGT GCA-3'	
	3377	3397
S4	5'-GCC ACC AAA GAG AAC CTG ATC-3'	
	3397	3377
AS4	5'-GAT CAG GTT CTC TTT GGT GGC-3'	
	3470	3490
S5	5'-GGA TTT AAC AGT GCG GGG GTC-3'	
	3490	3470
AS5	5'-GAC CCC CGC ACT GTT AAA TCC-3'	
	3521	3541
S6	5'-GGT CTT CGA CCC ACC GGA GTT-3'	
	3541	3521

AS6	5'-AAC TCC GGT GGG TCG AAG ACC-3'	
	3752	3772
S7	5'-CTT CAG CAC GGC AAA AAT GAG-3'	
	3772	3752
AS7	5'-CTC ATT TTT GCG GTG CTG AAG-3'	
	4070	4090
S8	5'-AGG CTT TCT AGA TCG AGC TCG -3'	
	4090	4070
AS8	5'-CGA GCT CGA TCT AGA AAG GCT-3'	
	4136	4126
AS9	5'-CTT CGA TTT TGG ATC CGT TGT-3'	

Site Directed Mutagenesis Primers

Arg70Leu

	3197	3229
S	5'-TAC TAC GAG GAG ATT <u>CTC</u> GGT ATT GCA AAG GGC-3'	
	3229	3197
AS	5'-GCC CTT TGC AAT ACC <u>GAG</u> AAT CTC CTC GTA GTA-3'	

Asn267Ser

	3788	3820
S	5'-TTA CCG GAC TCA TGG <u>AGT</u> CGC CAC CAG CGT ATG-3'	
	3820	3788
AS	5'-CAT ACG CTG GTG GCG <u>ACT</u> CCA TGA GTC CGG TAA-3'	

Chapter 4 pHAR4-p plasmid (8594bp) *penDE* (2761-3834)

Sequencing Primers

As for pHAR10a (primers S2, AS2, S3, AS3, S4, AS4, S5, AS5, S6, AS6, S7, AS7) plus the following;

	2051	2071
per1S	5'-TCA ACC TGC AAG AAC CGT ACT-3'	
	2701	2721
per2S	5'-ACT AAT TCG AGC TCG AAC AAC-3'	
	2721	2701
per2AS	5'-GTT GTT CGA GCT CGA ATT AGT-3'	
	4021	4000
per3AS	5'-TTG GGA AGG GCG ATC GGT GCG-3'	

Site Directed Mutagenesis Primers

Arg1Met

	2746	2779
S	5'-CTC GGG ATC GAG GGA <u>ATG</u> CTT CAC ATC CTC TGT-3'	
	2779	2746
AS	5'-ACA GAG GAT GTG AAG <u>CAT</u> TCC CTC GAT CCC GAG-3'	

Glu338Ser

	3757	3780
S	5'-CGG ACC AAC CCT GAT <u>TCG</u> ATG TTT GTC ATG CGG-3'	
	3780	3757
AS	5'-CCG CAT GAC AAA CAT <u>CGA</u> ATC AGG GTT GGT CGG-3'	

Chapter 5 pUC18 plasmid (2686bp) *bla* (201-1061)

Sequencing Primers

	96	116
blaS1	5'-CGC GGA ACC CCT ATT TGT TTA-3'	
	1127	1107
blaAS2	5'-CCT TTT AAA TTA AAA ATG AAG-3'	
	281	301
blaS3	5'-GCT GGT GAA AGT AAA AGA TGC-3'	
	2170	2151
blaAS4	5'-ATA CGA GCC GGA AGC ATA AAC-3'	

Site Saturation Mutagenesis Primers

Asn132Xxx

	573	605
S	5'-ATA ACC ATG AGT GAT <u>NNN</u> ACT GCG GCC AAC TTA-3'	
	605	573
AS	5'-TAA GTT GGC CGC AGT <u>NNN</u> ATC ACT CAT GGT TAT-3'	

Ala237Xxx

	888	920
S	5'-GCT GAT AAA TCT GGA <u>NNN</u> GGT GAG CGT GGG TCT-3'	
	920	888
AS	5'-AGA CCC ACG CTC ACC <u>NNN</u> TCC AGA TTT ATC AGC-3'	

Site Directed Mutagenesis Primers

Asn132Asp

	573	605
S	5'-ATA ACC ATG AGT GAT <u>GAC</u> ACT GCG GCC AAC TTA-3'	
	605	573
AS	5'-TAA GTT GGC CGC AGT <u>GTC</u> ATC ACT CAT GGT TAT-3'	

Appendix E *P. chrysogenum* vs *A. nidulans*

Comparison of amino acid sequences of the *penDE* genes from *Penicillium chrysogenum* and *Aspergillus nidulans* (Cheung 2006). Only the mismatches are shown for *Aspergillus nidulans*.

	1	10	20	30	40				
<i>P. chrysogenum</i>	MLHILCQGTPEEIGYEHGSAAKAVIARSIDFAVDLIRGKT								
<i>A. nidulans</i>	VT	S	H	GE	KA				
	41	50	60	70	80				
<i>P. chrysogenum</i>	KKTDEELKQVSQLGRVIEERWPKYYEEIRGIAKGAERDV								
<i>A. nidulans</i>	QA	E	L	RE	MKQ	R	C	E	
	81	90	100	110	120				
<i>P. chrysogenum</i>	SEIVMLNTRTEFAYGLKAARDGCTTAYCQLPNGALQGGQNW								
<i>A. nidulans</i>		VE		V	KT				
	121	130	140	150	160				
<i>P. chrysogenum</i>	DFFSATKENLIRLTIRQAGLPTIKFITEAGIIGKVGFNSA								
<i>A. nidulans</i>	T	Q	C	P	M				
	161	170	180	190	200				
<i>P. chrysogenum</i>	GVAVNYNALHLQGLRPTGVPSHIALRIALESTSPSQAYDR								
<i>A. nidulans</i>		H	L	L	M	E	EK		
	201	210	220	230	240				
<i>P. chrysogenum</i>	IVEQGGMAASAFIMVGNGHEAFGLEFSPTSIRKQVLDANG								
<i>A. nidulans</i>	S		A	Y	I	LC	A	T	
	241	250	260	270	280				
<i>P. chrysogenum</i>	RMVHTNHCLLQH GKNEKELDPLPDSWNRHQRMEFLLDGFD								
<i>A. nidulans</i>	I	N	PSAQ	N	S	G	H	S	
	281	290	300	310	320				
<i>P. chrysogenum</i>	GTKQAFAQLWADEDNYPFSICRAYEEGKSRGATLFNIIYD								
<i>A. nidulans</i>	E	K	E	L	K	S	VF		
	321	330	340	350	357				
<i>P. chrysogenum</i>	HARREATVRLGRPTNPDEMFMRFDEEDERSALNARL								
<i>A. nidulans</i>	VG	K	N	T	T	SNL	TK	IQ	NI

Appendix F *E. coli* Strains

XL1-Blue

recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB lacI^qZDM15Tn10 (Tet^r)].

XL1-Red

endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac mutD5 mutS mutT Tn10 (Tet^r)

Appendix G ^1H NMR Spectra for Chapter 4